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on myeloid Cells-2 in bacterial Lung Infections

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1. Abstract

Pneumonia caused by *Streptococcus pneumoniae* (*S. pneumoniae*) is worldwide an important healthcare concern amongst young, old and immunosuppressed patients. Alveolar macrophages (AMs) are the first line of defense, as they protect the lung from various pathogens and are crucial for the maintenance of lung homeostasis. The triggering receptor expressed on myeloid cells-2 (TREM-2) is a surface receptor, which is expressed on AMs and many other myeloid cells. Next to the role in bone formation and brain function, TREM-2 seems to be a negative regulator of immune responses. This is in contrast to a closely related receptor called TREM-1, which was shown to amplify immune responses. These contrasting effects are of special interest because both receptors require the DNAX activating protein of molecular mass 12 kDA (DAP12) for signaling. Considering that Toll-like receptors (TLRs) as well as TREM-2 are constitutively expressed on lung mononuclear phagocytes and that these cells are crucial in the modulation of the inflammatory response upon infection, we hypothesize that TREM-2 plays an important role during bacterial pneumonia.

In my work I show, that TREM-2 deficient AMs show enhanced phagocytosis of *S. pneumoniae* compared to wildtype cells. One possible explanation for this might be elevated Phosphatidylinositol 3 kinase (PI3-Kinase) activity, as well as increased ERK activity in the TREM-2 knockout cells. Furthermore I show evidence for the role of TREM-2 in the regulation of the actin cytoskeleton as well as for the regulatory function of TREM-2 in cytokine production.

Another aspect of my diploma project was the identification of interactors downstream of TREM-2 or TREM-2/DAP12. We for this purpose decided to perform pulldown experiments and it was my task to make a TREM-2/DAP12 fusion construct and to generate stably transfected cell lines, expressing N-terminal tagged TREM-1, TREM-2 or the chimeric construct. The respective data are presented in this thesis.

2. Zusammenfassung

Pneumonie, meistens verursacht durch *Streptococcus pneumoniae*, ist ein weltweites Gesundheitsproblem und betrifft alle Altersgruppen sowie immunsupprimierte Patienten. Alveolarmacrophagen (AMs) sind die wichtigsten Zellen des angeborenen Immunsystems der Lunge in frühen Phasen einer Infektion und außerdem notwendig für die Erhaltung des homöostatischen Gleichgewichts. Triggering receptor expressed on myeloid cells-2 (TREM-2) ist ein Oberflächenezeptor der von AMs und anderen myeloischen Zellen exprimiert wird. Neben seiner Rolle in Knochenbildung und Gehirnfunktion, ist TREM-2 ein wichtiger Negativregulator von Immunantworten, im Gegensatz zu dem nahe verwandten Rezeptor TREM-1, einem Verstärker von Entzündungsreaktionen. Dieser Kontrast ist interessant, da beide Rezeptoren mit dem gleichen Adaptorprotein, DAP12, assoziieren. Da TLRs sowie TREM-2 konstitutiv von mononuklearen Phagozyten der Lunge exprimiert werden und essentielle Faktoren für die Modulation von Entzündungsreaktionen sind, gehen wir davon aus, dass TREM-2 eine wichtige Rolle in der Pathogenese einer bakteriellen Pneumonie spielt.

In dieser Arbeit verifiziere ich, dass TREM-2^{-/-} AMs eine verstärkte phagozytische Aktivität haben, wenn sie mit *S. pneumoniae* stimuliert werden. Die Zellen zeigen außerdem eine erhöhte PI3-Kinase Aktivität sowie erhöhte ERK Aktivität. Weiters gibt meine Arbeit Hinweise auf eine regulatorische Funktion von TREM-2 für die Organisation des Aktin-Zytoskeletts sowie für die Cytokinproduktion. Der Fokus dieser Arbeit lag auf der Herstellung eines TREM-2/DAP12 Fusionskonstrukts und der Generation von stabil transfizierten Zelllinien, die N-terminal getaggte TREM-1, TREM-2 oder das Fusionskonstrukte exprimieren. Das Ziel war ein Pulldown Experiment um mögliche intrazelluläre Interaktionspartner von TREM-2 zu identifizieren.

3. Diplomathesis and research question

Since function and downstream signaling of TREM-2 is not well understood, the aim of this diploma was, to further investigate the role of TREM-2 in pneumococcal infection, with a special focus on the signal transduction mediated by TREM-2. One approach was to compare the response of AMs from WT and TREM-2 deficient mice, upon stimulation with *S. pneumonia* and Lipopolysaccharide (LPS) in order to find possible differences in the activation of several pathways. Since morphological changes of the actin cytoskeleton were observed in TREM-2 deficient DCs and osteoclasts, confocal analysis of WT and TREM-2^{-/-} AMs was performed.

Another aim was to elucidate DAP12 dependent downstream signaling pathways induced via TREM-2 and to find novel interaction partners of TREM-2/DAP12 as well as possible downstream mediators of the TREM-2/DAP12 signaling complex upon stimulation with *S. pneumoniae*.

To answer this question the idea was to produce stable TREM-2 transfected cell lines and perform a pulldown in Flp-InTM T-RExTM HEK 293 cells, by using a Streptavidin-Hemagglutinin-tag (SH-tag), as well as in RAW 264.7 macrophages, using a Tandem affinity purification system with a tag consisting of Protein G coupled to streptavidin binding protein (GS-tag). The pulldown in HEK cells was performed because they are easy to transfect and the construct is not overexpressed, because it is inserted in the HEK cell genome just once. The pulldown in the RAW cells has the disadvantage that transfection of these cells is not so easy and a retroviral transfection method has to be used, causing random insertion and overexpression of the constructs. Despite this a TREM-2 pulldown in RAW cells is of special interest, because of the possibility to study differences in complex formation when the cells are stimulated with *S. pneumoniae*. This is not possible in HEK cells because they do not express TLRs and therefore do not respond to the stimulus.

Because the intracellular domain of TREM-2 is quite small, the proteins were tagged on the the N-terminus. Another problem is that many components of the TREM-2/DAP12 complex might only bind to TREM-2 associated DAP12 and might not get

pulled down with TREM-2. To overcome this problem I generated a TREM-2/DAP12 chimeric construct, which consists of the extracellular domain of TREM-2, fused to the intracellular domain of DAP12. Since there might be the possibility that the transmembrane part of DAP12 can pair with other receptors, like e.g. TREM-1, dectin-1, FcyR and thereby falsify results, I also generated a construct in which I introduced a mutation in the transmembrane (TM) domain of the chimera (TM-mutant chimera) that inhibits pairing with competing receptors (Fig. 1).

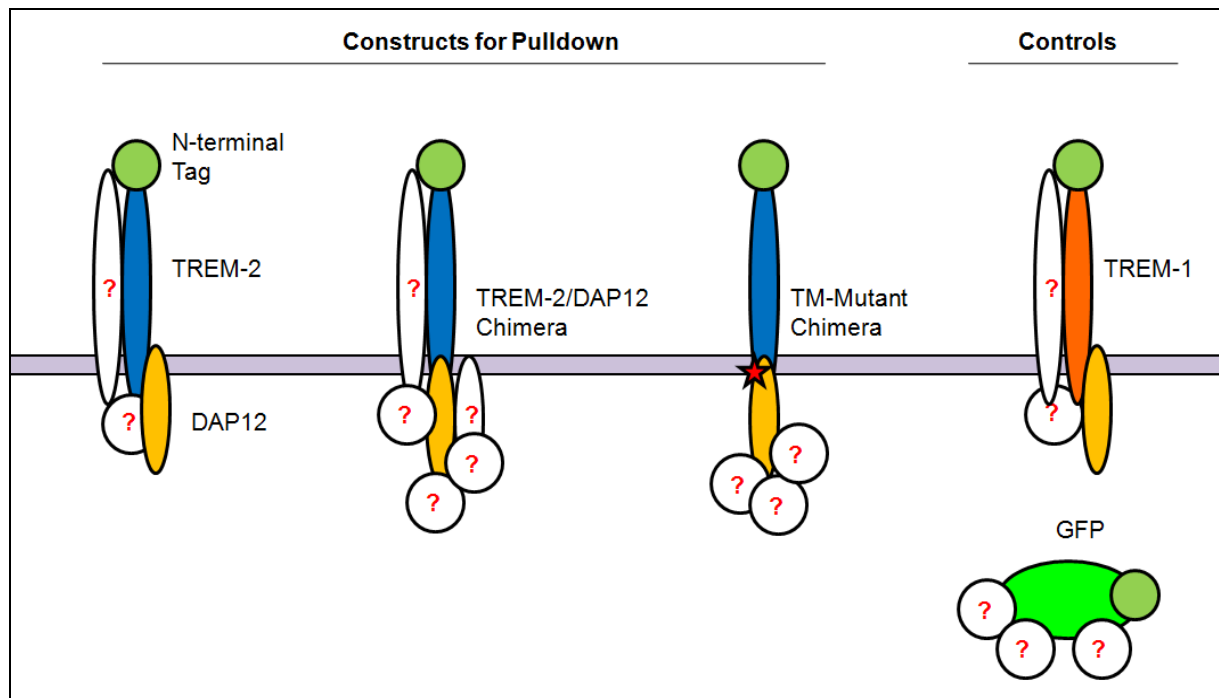


Fig.1: Pulldown constructs and controls: TREM-2, TREM-2/DAP12 chimera (Chimera), transmembrane mutant TREM-2/DAP12 chimera (TM-mutant chimera)

This approach might help to find possible adaptors, other than DAP12 which should interact with TREM-2 within the membrane, as well as binding partners of the small cytoplasmic domain of TREM-2. The TM-mutant of the TREM-2/DAP12 chimera functions as an important control and could pulldown proteins which interact specifically with the TREM-2/DAP12 complex, other controls will be TREM-1 as well as the exogenous protein GFP, which should allow to identify unspecific binding partners. By comparing pulldowns of stimulated and unstimulated cells this study should help to get insights into the function of TREM-2 upon infection.

4. Introduction

4.1. Innate immunity and inflammation of the lung

The mammalian immune system can be viewed as consisting of 3 different levels, anatomic and physiological barriers, the innate immune system and the adaptive immune system (Fig. 2) [1].

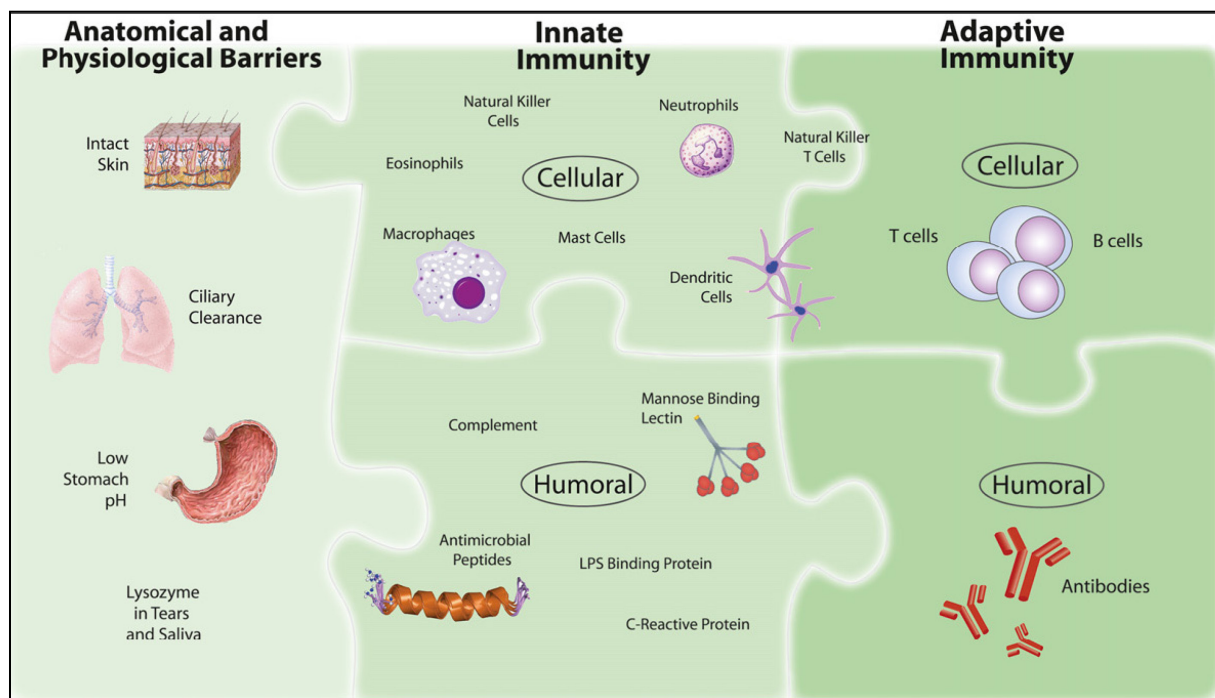


Fig.2: Integrated human immune system (modified from Turvey and Broide 2010) [1]

The adaptive immune system is mediated by T- and B-Lymphocytes which are able to randomly generate a massive repertoire of antigen receptors against small and highly specific structures, called epitopes [2]. In contrast to the adaptive immune system, the innate immune system relies on a limited, germ line encoded repertoire of receptors, targeting conserved microbial structures that are shared by whole groups of pathogens and is mediated by cells of both hematopoietic and non-hematopoietic origin [3, 4]. The innate immune system is very fast. It reacts within minutes after antigen exposure and starts to generate an inflammatory response. In

parallel with this initial inflammatory response, it is the innate immune system that activates and tailors the subsequent adaptive immune response [1].

The function of the lung is to supply the body with oxygen and to release carbon dioxide. This happens in the lung alveoli, which are made up of small type-I alveolar epithelial cells (AECs) on the surface and the more cuboidal type-II AECs, which are necessary for the production of surfactant and have self-renewal and differentiation potential. Since the inhaled air contains many particles including potential pathogenic microorganisms, the large surface of the lung also works as a “filter”. Lung capillaries are close to type I AECs to allow a proper gas exchange, but because of this proximity the lung is also an entrance point for many pathogens to enter the bloodstream, where they could cause a systemic infection. To avoid permanent inflammation, the immune system of the lung has to be highly regulated [5, 6].

The lung is protected from potential harmful microorganisms by nonspecific and specific defense mechanisms. The resident immune system of the lung includes mucociliary clearance, antimicrobial proteins in the airway surface liquid and populations of myeloid cells. These cells function as sentinels and they are well equipped with pattern recognition receptors, strategically located in the lung tissue to encounter microbes in the air space [1]. The outcome of a pulmonary tract infection is dependent on two factors, the pathogen and the ability of the host to fight the infection, by eliciting an effective immune response. Small numbers of microbes might be cleared by the resident immune system, but numerous microbes or a higher virulent strain cause an inflammatory response [1].

Resident alveolar macrophages (AM) have been shown to be the first line of cellular defense and they are very important in the early phases of an infection. They are crucial for the maintenance of lung homeostasis and the modulation of the immune response [1, 7]. Only when the nonspecific mechanisms fail to eliminate a pathogen, pulmonary dendritic cells (DC) get activated and elicit an adaptive immune response. Next their key role in activating the adaptive immune response, DCs also have an important innate immune function in the response to viruses, by producing high levels of type-I-interferons [8].

In case of a serious infection, these early events lead to the recruitment of neutrophils, the major effector cells of innate immunity. They migrate out of the pulmonary capillaries and accumulate in the air spaces. After phagocytosis, neutrophils kill the ingested microbe with reactive oxygen species (ROI, NO), antimicrobial proteins and proteases [7, 8].

Lung inflammation can cause tissue damage and seriously harm the host. Thus, to better understand the interactions between host and pathogen during pneumococcal pneumonia, it is crucial to further investigate the mechanisms, which are determining the intensity of an inflammatory response in the lung.

To sum up, inflammation always has two faces and it is of particular importance that inflammatory responses are highly regulated in the lung. A weak inflammatory reaction might not clear the host from a pathogen, whereas overwhelming inflammation directly contributes to the damage of cells and tissues. Thus lung injury and pulmonary dysfunction during infection are the result of a harmful interaction between the host and the pathogenic species [1, 6].

4.1.1. Pathogen recognition by the innate immune system

As mentioned above, macrophages and all other cells of the innate immune system recognize infectious and endogenous ligands via so called pattern-recognition receptors (PRRs). PRRs are germ line encoded receptors, which bind to highly conserved molecular structures, known as pathogen-associated molecular patterns (PAMPs) [3, 4]. When PRRs get ligated, they trigger the release of proinflammatory cytokines, like tumor necrosis factor- α (TNF α), interleukin-6 (IL-6), interleukin-1 β and type I interferons (IFNs)[9] and induce the activation of the adaptive immune response[10].

Today, many different classes of PRRs are known to be important for the immune response. These are the Toll-like receptors (TLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) or the Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [11].

Twelve members of the TLR family have so far been identified in mammals, and the ligands for 11 TLRs are known. TLRs are glycosylated type I integral membrane proteins which form homodimers, like TLR3-TLR3, or heterodimers, like TLR1-TLR2 or TLR2-TLR6. Signal transduction occurs upon ligation via their cytosolic Toll/IL-1 receptor-like domain (TIR). TLR signaling leads to the activation of the nuclear factor κ B (NF κ B), the interferon regulatory factors (IRF) and other transcription factors, which ultimately results in the production of proinflammatory cytokines and other immunologic responses, like the maturation of DCs (Fig. 3) [1]. NF κ B proteins (also called Rel transcription factors) play key roles in the regulation of the innate as well as of the adaptive immune response. Under normal conditions they are located in the cytoplasm in association with inhibitory proteins called the inhibitors of NF κ B (I κ B). Upon stimulation of a cell, I κ Bs get phosphorylated, which is followed by proteasomal degradation. Thereby NF κ B is released and can translocate to the nucleus, where it binds to the DNA and activates various genes [12]. Some TLRs are expressed on the cell surface (TLR1, 2, 4, 5, 6), while others are expressed intracellular in endosomal compartments, like the endoplasmatic reticulum (ER) or endosomes (TLR3, 7, 8, 9). For a complete overview about TLRs, their localization and their ligands see Kumar 2009 [11].

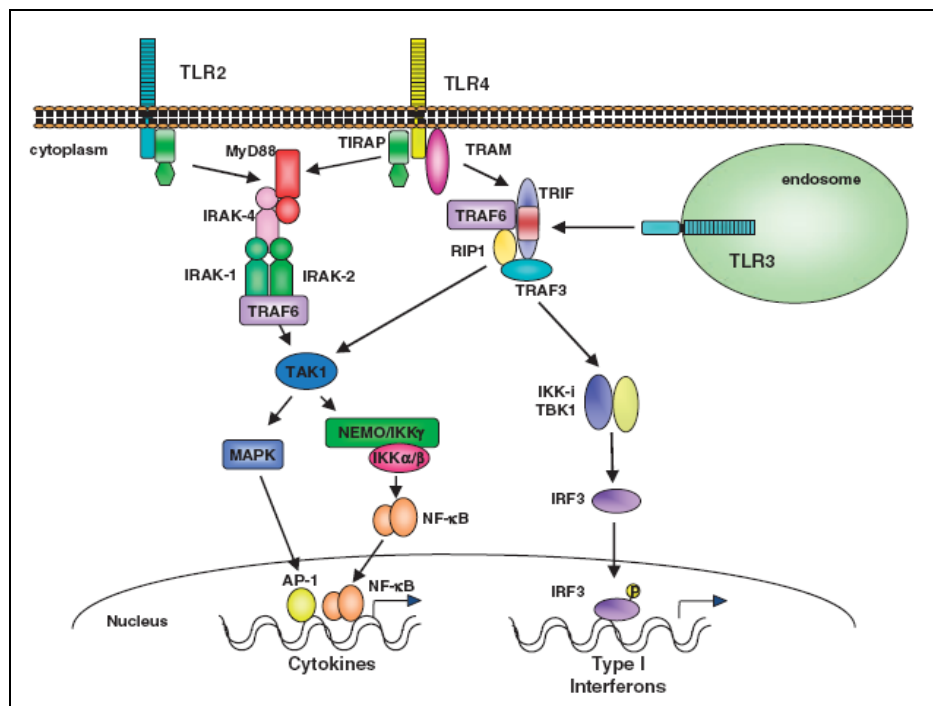


Fig.3: MyD88- and TRIF-dependent pathways in TLR signaling (modified from Akira 2009) [13]

Another PRR on phagocytes is the C-type lectin family member dectin1. Dectin1 is a transmembrane receptor with an important role in antifungal immunity, which can recognize the polysaccharide β -glucan that is part of the fungal cell wall. Upon stimulation, the immunoreceptor tyrosine-based activation motif (ITAM) of dectin1 gets phosphorylated and recruits the protein tyrosin kinase Syk, which then activates a signaling pathway involving CARD9, Bcl-10 and MALT1 [14, 15].

Nod-like receptors (NLRs) are a large family of about 20 proteins, which have diverse functions mediated by their N-terminal domain. All NLRs contain a nucleotide-binding oligomerization domain (NOD) and can be categorized in three subfamilies depending on their N-terminal domain. This domain is either a caspase-recruitment domain (CARD), in members of the NOD subfamily, a pyrin domain in the NALP subfamily (proteins containing a NACHT domain, leucine rich repeats and a pyrine domain) or a baculoviral-inhibitor-of-apoptosis-protein-repeat-containing (BIR) domain, in the NAIP (neuronal apoptosis inhibitory protein) subfamily [16, 17]. Members of the NOD subfamily, NOD-1 and NOD-2 are involved in the recognition of bacterial peptidoglycans and can trigger the production of cytokines and chemokines as well as the recruitment of neutrophils to the site of infection [17]. It has also been shown that they are important for the initiation of the adaptive immune response [18]. The NALP and the NAIP subfamilies are important for the production of IL-1 family members, like IL-1 β . These cytokines are produced as inactive precursor molecules that need to be cleaved by caspase 1. In contrast to other cytokines, which require only TLR activation, IL-1 release therefore requires a second signal, which results in the activation of the inflammasome. Inflammasomes are large protein complexes, categorized according to their composition (NALP or NAIP), which are able to induce cleavage of pro-caspase 1, which in turn leads to processing of IL-1 precursor proteins [17, 19, 20].

Two types of cytosolic PRRs are known to be responsible for the recognition of viral nucleic acids. RIG-I and MDA5 are sensing viral RNA, whereas viral DNA is recognized by a protein called DAI [3, 17]. Since nucleic acid sensing PRRs are not of interest for the topic of this thesis and it would exceed the frame of this work, for more details see ref. 3 and 13.

4.1.2. Pneumonia

As a major cause of death worldwide and the 6th common cause of death in the United States, pneumonia is a serious, persistent and widespread public health problem. Community-acquired pneumonia (CAP) is the single most prevalent infectious disease in the world and is defined as pneumonia acquired outside of a hospital [8, 21, 22]. CAP can be caused by a variety of pathogens including gram-positive and gram-negative bacteria, viruses as well as fungi or protozoa. However, the single most frequent cause of CAP worldwide is based on an infection with *S. pneumoniae* [22].

Because of the high mortality of CAP and the growing problem of bacterial resistance to many commonly used antibiotics [22], research in this field is of great medical and also social interest. Since the outcome of any infection depends on the virulence of the organism and the immune response of the host it is necessary to further understand the molecular interactions between the invading microbe and the immune system of the host during the manifestation of disease.

4.1.2.1. *Streptococcus pneumoniae*

S. pneumoniae is a gram-positive bacterium and one of the most important human pathogens. It colonizes the human nasopharynx and is the most frequent cause of pneumonia but can also lead to meningitis or otitis media. A vaccine against pneumococci is available, but it is not protective against all serotypes and is not very efficient for very young individuals and older people [23]. Similar to other pathogens, the virulence of *S. pneumoniae* strains depends on the expression of various virulence factors, which are either specific escape mechanism to evade from the host immune recognition or toxins, which are directly harming the host.

The most important virulence factor is the polysaccharide capsule of *S. pneumoniae* with its strong antiphagocytic activity. The capsule shields the bacterial surface from binding of the complement component C3i and immunoglobulines. Thereby it prevents the interaction with corresponding Fc or complement receptors on

phagocytic cells and hence inhibits phagocytosis [24]. Furthermore, the polysaccharide capsule also enhances colonization, restricts autolysis, protects the pneumococcus against antibiotics [25] and significantly reduces trapping of the microbe within neutrophil extracellular traps [26].

The best studied pneumococcal virulence factor is the toxin pneumolysin (Ply), which has at least two biological activities, a lytic activity, by pore formation and the ability to activate the complement system of the host [23, 27].

Other virulence factors are LPXTG-anchored proteins, like hyaluronidase, neuraminidase or the serine protease PrtA. Important for the ability of *S. pneumonia* to colonize the human mucosa are several adhesive properties, which include pili and different lipoproteins. Choline-binding proteins (CBPs) are necessary for autolysis and thereby enhance the release of highly inflammatory cell wall degradation products as well as the release of pneumolysin. The pneumococcus also produces peptidoglycan modifying enzymes, making the pneumococcal peptidoglycan resistant to human lysozyme [25, 28], and expresses a DNase, which protects the bacteria from killing by neutrophils [26].

In this work, the capsulated, ply-positive serotype 3 strain ATCC 6303 was used for all experiments.

4.1.2.2. *Innate Immunity in pneumococcal pneumonia*

When a pneumococcus enters the lower respiratory tract, the bronchial and lung epithelium act as a mechanical barrier and epithelial cells as well as alveolar macrophages recognize the invader via their PRRs.

It is known that TLRs are crucial for the host to fight an infection with *S. pneumonia*. Pneumococcal cell wall components, like lipoteichoic acid (LTA) [29] and peptidoglycan, are recognized by TLR2 [30, 31] while ply is detected by TLR4 [32] and TLR9 might be involved in sensing bacterial DNA, released after autolysis of pneumococci [33].

TLR ligation by the pneumococcus induces signaling pathways, activating NF κ B and the IRF family of transcription factors [8]. TLR2 is expressed on Type-II AECs and has been shown to be responsible for *S.pneumoniae*-induced activation of the NF κ B proteins RelA and p50 in this celltype, which are required for proper cytokine production, neutrophil recruitment and killing of bacteria [34]. However, NF κ B has not only proinflammatory properties. While the translocation of RelA to the nucleus induces the expression of chemokines and adhesion molecules that are required for neutrophil recruitment, nuclear translocation of p50 downregulates the expression of pro-inflammatory genes [35, 36]. Translocation of NF κ B proteins in pneumonia is also induced by the early phase cytokines TNF α and IL-1 [12]. In a mouse model of pneumonia, TNF- α is one of the first cytokines which is found in the BAL fluid as well as in the tissue [7]. Although TNF- α is required to elicit a proper immune response, exaggerated expression can lead to overwhelming inflammation and subsequent lung injury, which increases the risk of septic shock and death. AMs are the main source of TNF- α and therefore, are important modulators of inflammation in pneumococcal pneumonia [30].

Although AMs are essential for phagocytosis during early phases of the disease, the most important phagocytic cells to clear the lung from bacteria and to limit bacterial dissemination are neutrophils [37]. The innate immune response not only recruits neutrophils to the lung, it also increases neutrophil generation in the bone marrow and shortens their maturation time. The cytokines TNF- α and IL-1 induce the expression of the chemokine IL-8 (corresponding to KC in mice), which is a crucial factor for neutrophil chemotaxis [7]. After neutrophils phagocytosed bacteria, they rapidly undergo apoptotic cell death and are eliminated by AMs [37]

Another important cytokine in pneumococcal pneumonia is IL-6. IL-6 increases the lifetime of neutrophils and enhances their cytotoxicity. Furthermore, IL-6 induces acute phase proteins such as C-reactive protein (CRP), and is involved in adaptive immune mechanisms [38]. The acute phase proteins CRP and mannose-binding lectin (MBL) are produced in the liver during pneumococcal pneumonia. They work as opsonins and enhance opsonized phagocytosis by neutrophils after binding to the bacterial surface [7, 39]. Together with the natural nonspecific immunoglobulin M (IgM), they activate the classical complement pathway [7].

To prevent exaggerated tissue damage, the inflammatory process has to be tightly regulated. Two important anti-inflammatory, regulatory cytokines are working in concert to limit inflammation [40]. These are interleukin-10 (IL-10) and transforming growthfactor- β (TGF β). IL-10 is crucial to reduce the production of proinflammatory mediators and can be produced by different subsets of helper- and regulatory T-cells, B-cells, granulocytes [41], neutrophils [42] as well as by AMs [5]. At baseline conditions TGF- β is expressed by AMs, counterbalances the activation of AMs and is therefore necessary to maintain lung homeostasis [5, 40].

4.2. Macrophages in innate immunity

4.2.1. Macrophage development

Macrophages are resident phagocytic cells found in lymphoid and basically all non-lymphoid tissues. They are leukocytes and belong to the system of mononuclear phagocytes (together with dendritic cells and monocytes). Monocytes circulate in the blood and populate different tissues where they differentiate into macrophages. Macrophages show remarkable heterogeneity, depending on their specific function within the tissue (Fig. 4) [43].

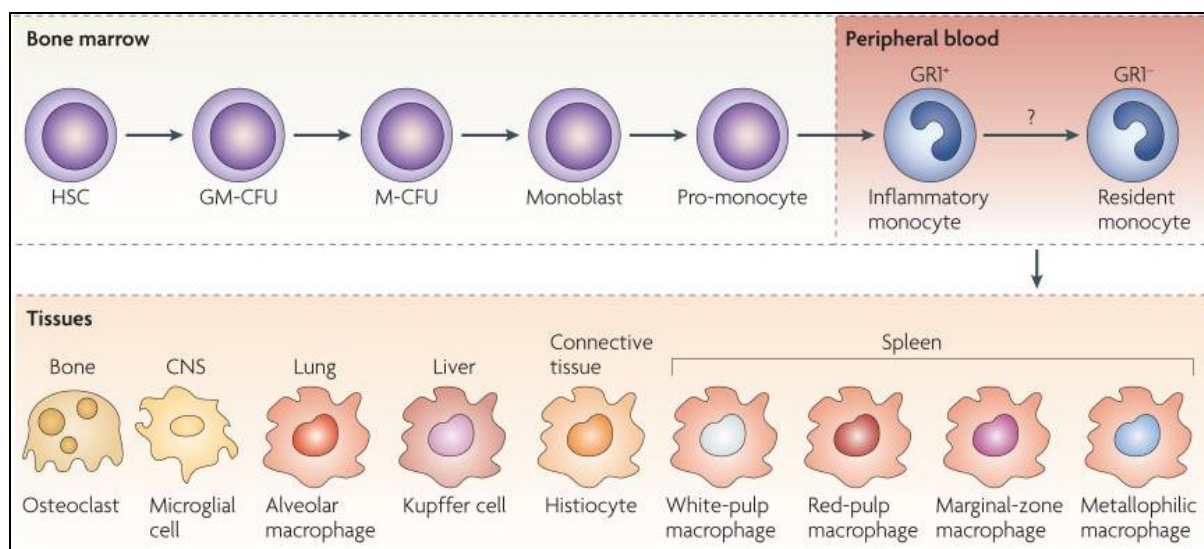


Fig.4: Monocyte heterogeneity (modified from Mosser 2008) [44]

In the bone marrow hematopoietic stem cells (HMCs) produce myeloid progenitor cells (MP) and lymphoid progenitor cells (LP). The common MPs give rise to monocytes and many other cell types, including neutrophils, eosinophils, basophils, DCs and mast cells [44]. Monocytes can be proinflammatory and are the main source of inflammatory macrophages in tissues during an inflammatory process or trauma. Next to this, fully matured macrophages can also develop from tissue-resident colony-forming cells, like it is the case for microglia [43].

4.2.2. Macrophage function and polarization

Macrophages are involved in immune responses, but they also have essential vital homeostatic roles. During an immune reaction they are crucial phagocytic, killing and antigen presenting cells and furthermore produce cytokines which are important to modulate the immune response. In a steady state situation macrophages are important in the removal of cellular debris during tissue remodeling and they clear the organism from apoptotic cells. In general, the receptors which are involved in these immune-cell independent processes fail to induce cytokine production or actively produce inhibitory signals [44].

When macrophages get activated, they can develop different functions and properties, depending on the stimulus. Endogenous stimuli are generated by innate immune cells, antigen-specific immune cells or from macrophages themselves. Exogenous stimuli are mostly PAMPs. In the past, macrophages have been classified into two groups, classically activated macrophages (M1) and alternatively activated macrophages (M2) [45]. Today it is known that there are much more types of macrophages and the term M2 has been changed to include all types of a macrophage, other than classic M1 [44, 46]. To get an overview, three major macrophage populations will be shortly mentioned in this work.

Classic activation of macrophages happens due to stimulation with $\text{IFN}\gamma$, $\text{IFN}\beta$ or $\text{TNF}\alpha$. In general, TLR agonists, like LPS, induce this phenotype, which is characterized by a high capacity to present antigen, produce proinflammatory cytokines ($\text{IL-12}^{\text{high}}$) and to generate toxic intermediates (NO, ROI) [44, 46].

Stimulation with IL-4 and/or IL-13 leads to the development of wound-healing macrophages. These M2 have an enhanced arginase activity, an enzyme that converts arginine to ornithine, which is a precursor of polyamines and collagen and thereby enhances the production of extracellular matrix. These macrophages produce only small amounts of proinflammatory cytokines and have decreased microbicidal activity, compared to classic M1 macrophages [45-47]. Regulatory macrophages are a sub-population of M2, which differentiate upon stimulation with glucocorticoids and/or immune complexes. These macrophages are characterized by a high production of the regulatory cytokine IL-10, very low expression of proinflammatory cytokines, a low capacity to present antigen and a low microbicidal activity. It is believed that their main function is to dampen an immune response and to limit inflammation [44, 47, 48].

4.2.3. Alveolar macrophages

Alveolar macrophages are resident macrophages in the lung and are crucial to maintain lung homeostasis. Since AMs are continuously encountering inhaled substances, they have to be kept in a quiescent state to avoid tissue damage due to overwhelming inflammation. Thus, AMs have to be able to distinguish between harmless and potentially dangerous antigens and for a long time it was unknown which mechanisms are involved in the maintenance of lung homeostasis and which signals are required to induce an immune response.

In steady state situations AMs produce low amounts of proinflammatory cytokines and show poor phagocytic activity. When they are mixed with DCs in vitro, they successfully suppress T-cell activation by producing IL-10, prostaglandins and TGF- β [5]. Furthermore AMs actively suppress other innate and adaptive immune responses[49]. Under non-infectious conditions, AMs are in close contact with type-I and type-II AECs, which enable them to express the integrin $\alpha v \beta 6$ that activates TGF- β . When AMs encounter danger signals like TLR ligands, they lose the close contact to the AECs and $\alpha v \beta 6$ gets downregulated. This leads to a loss of TGF- β inhibition, and AMs get activated, start to produce proinflammatory cytokines and

show enhanced phagocytic activity together with an increased killing efficiency. After a while the macrophages themselves reactivate TGF- β and go back to the steady state situation [50].

4.2.4. Phagocytosis

Phagocytosis is defined as the actin dependent uptake of large particles (bigger than 0.5 μ m diameter) by leukocytes and plays a very important role in the host defense against various invading microorganisms and the removal of apoptotic cells [51].

Two different receptor dependent mechanisms can mediate the internalization of particles by macrophages or neutrophils. On the one hand binding of a particle to a receptor can stimulate the extension of pseudopodia, which are then surrounding the particle and fuse at their tips. On the other hand a receptor can induce tight binding of the particle to the membrane, which is then displaced inwardly, by sinking the target into the phagocytic cell [52]. In both cases the particle ends up inside of the cell and gets processed in the so called phagosome, a membrane bound vacuole, which undergoes maturation. Maturation happens by finely tuned remodeling of the vacuole by fusion and fission with early and late endosomes as well as lysosomes. Especially late endosomes and lysosomes provide a lot of microbicidal components and contribute to the stepwise acidification of the phagosome, which finally results in the hybrid-organelle, called the phagolysosome.

There are currently two alternative models, which try to explain how phagocytes can ingest large or multiple particles, without significant loss of cellular surface area. One hypothesis, the conventional model, postulates that the permanent fusion of endosomes with the plasmalemma keeps the surface area constant. The new hypothesis, the ER-mediated model, describes phagosome formation as a result of sliding of the particle into the endoplasmatic reticulum via an opening formed at the base of the phagocytic cup. In this case the ER provides most of the membrane to form the phagocytic vacuole, with maximal conservation of the surface membrane [53].

4.2.5. Signaling in phagocytosis

The initiation of the uptake of a particle and the activation of the phagocytosis machinery is triggered by multiple binding and clustering of phagocytic receptors. Today many phagocytic receptors on mammalian cells are known to be involved in the innate immune response. Examples for important receptors on macrophages are the different complement receptors (CRs), which bind to complement opsonized bacteria or fungi, the Mannose receptor, Fc γ Rs, scavenger receptors like MARCO or CD36 and many others [54].

Activation of these receptors recruits members of the Src family, which either leads to phosphorylation of the receptors themselves, or to phosphorylation of immunoreceptor tyrosine-based motif (ITAM)-containing subunits [55]. The exact order of events after receptor activation is not clear. Phosphorylated ITAM then recruits the kinase Syk [56] and/or other kinases and the formation of the activated receptor complex involves recruitment of further adaptor molecules. Finally activation of the Phosphatidylinositol 3-kinase (PI3K) leads to lipid remodeling, by accumulation of 3'-phosphoinositides. Lipid remodeling is a highly regulated process and restricted in space and time, and at least partly controlled by the lipid phosphatase SHIP, which is also recruited to the phagocytic cup [57]. Phagocytosis leads also to an increased synthesis of phosphatidylinositol-4,5-bisphosphate (PIP₂), which is a substrate of the PI3K but also of phospholipase C (PLC), which produces diacylglycerol (DAG). DAG in turn activates protein kinase C (PKC). In addition, other kinases like MEK1, ERK or the protein kinase A (PKA) have been shown to get activated upon phagocytosis [54]. This whole activation cascade goes hand in hand with cytoskeletal rearrangements, like the accumulation of F-actin and associated proteins in the periphagosomal region, which is mediated by different members of the Rho family of GTPases [58, 59] and leads to extension of pseudopodia.

4.3. Triggering receptor expressed on myeloid cells-2 (TREM-2)

4.3.1. The TREM receptor family

TREM proteins are a family of surface receptors, which mediate many different processes, like bone formation, neurological development and inflammation. The TREM cluster is located on the human chromosome 6p21 and on the mouse chromosome 17C3 and includes the genes encoding for TREM-1 and TREM-2 in both species, TREM-3, TREM-5 and a recently identified member PDC-TREM in mice, as well as genes encoding for the so called “TREM like” transcripts (TLTs) [60, 61]. All members of the TREM family comprise single IgSF V-type domains. TREM-1, TREM-2 and TREM-3 do not contain any known motifs for signal transduction and signaling requires association with DAP12, an immunoreceptor tyrosine-based activating motif (ITAM) containing adaptor molecule. In contrast, some of the TLTs contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which have been shown to recruit the phosphatases SHP-1 and SHP-2 [62, 63].

Members of the TREM family are expressed on a variety of myeloid cells, where they mediate many diverse activating and inhibitory functions. The expression of TLT-2 on B- and T-lymphocytes is the only reported case of expression in the lymphoid lineage of cells [61]. One important feature of the TREM family is the ability to release soluble forms. Today soluble TREM-1, TREM-2 and TLT-1 have been detected in biological fluids of humans and animals suffering from infections [61]. It is not known now how these soluble receptors are generated, there is evidence for alternative splicing, but also for metalloproteases which cut the membranebound form [61].

TREM-2 is an orphan receptor, expressed on myeloid cells as well as on non-myeloid cells [64]. Like other TREM family members the extracellular part of TREM-2 comprises an IgSF V-type domain. It has a short cytoplasmic tail, a single pass, positively charged transmembrane (TM) domain and does not contain any known motifs for signal transduction [60]. Although a crystal structure for TREM-1 has been determined at 1.47Å [65], 1.76Å [66] and at 2.6Å [67] resolution, the crystal structure of TREM-2 is still unknown.

4.3.2. Activating and inhibitory functions of DAP12

To investigate TREM-2 signaling it is necessary to better understand the signal transduction mediated by its adaptor protein DAP12. Inside of a cell, downstream signals from many different receptors are overlapping and interacting in either a synergistic or an antagonistic way. Synergistic pathways are necessary to maintain the system sensitive to several stimuli, which individually would not reach the threshold to elicit cell activation. Antagonistic pathways are crucial to increase the specificity of a response by limiting activation in the presence of inhibitory signals [68].

It is well known, that surface receptors with diverse functions associate with ITAM-containing adaptor proteins like DAP12 or FcR γ . DAP12, also known as TYROBP or KARAP, seems to play a key role in signal integration within a cell. DAP12 was first described to mediate NK-cell activation via association with the killer-cell immunoglobulin like receptor (KIR) family and since this time many more cell surface receptors have been found to require pairing with DAP12 for signal transduction, like the Ly49 family on NK-cells and some T-cells, MDL1 on myeloid cells and many others. Also many members of the TREM family signal via DAP12 [68]. For a more detailed list of DAP12 associated receptors see Lanier 2009 or Turnbull 2007 (Fig. 5) [68, 69].

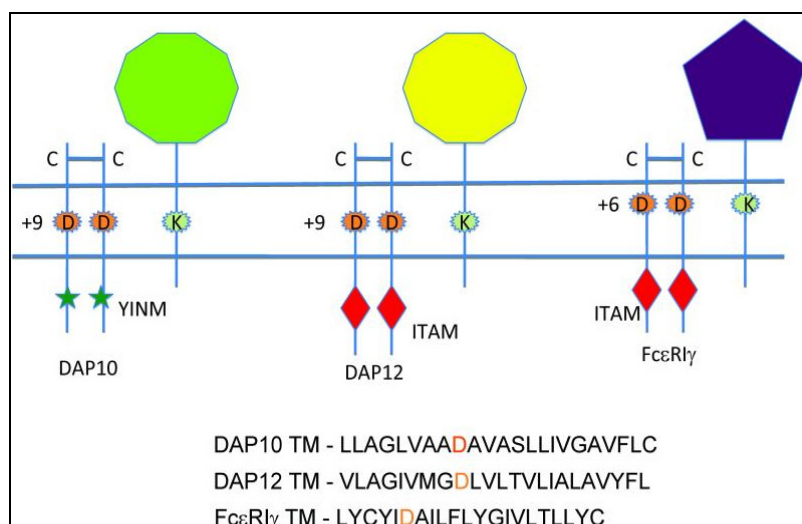


Fig.5: positively and negatively charged residues within the transmembrane domains of DAP10, DAP12, FcR γ and their associated receptors (modified from Lanier 2009) [69]

DAP12 is a short peptide with a length of only 113-114 amino acids, that has a minimal extracellular region (13-17 amino acids), a single pass transmembrane domain and a cytoplasmic domain, which comprises an ITAM [68], a conserved signaling element, which is defined as the following sequence D/ExxYxxL/Ix₆₋₈YxxL/I [69].

It has been shown that DAP12 interacts with associated receptors within the transmembrane domain via aspartic acids, which are centrally located within the TM-domain of DAP12. Thus, the location of positively charged residues like lysine or arginine is critical for a receptor to pair with DAP12 and TREM-2 contains such a Lysin (Fig.4) [70].

Although DAP12 was first found as a mediator for cellular activation, over the last years inhibitory functions have been shown to be mediated by DAP12 as well [69, 71, 72].

4.3.2.1. *The ambiguity of ITAM signaling*

To understand the ambiguous character of DAP12 it is necessary to explain more about ITAMs and ITIMs. Based on their names, it was long believed that ITAMs always mediate activation, while ITIMs mediate cellular inhibition. Confusingly, further research identified ITAMs to exert inhibitory functions and ITIMs to mediate activating functions [73].

Upon ligation of any DAP12-associated receptor, Src family kinases get activated which then phosphorylate tyrosin residues within the ITAM of DAP12. Dual phosphorylated ITAMs serve as docking sites for the tandem SH2-domains of Syk kinases (Syk and/or Zap70), leading to phosphorylation of various substrates and subsequent cellular activation [68]. Inhibitory DAP12 signaling is less well understood. ITIMs contain the consensus sequence S/I/V/LxYxxI/V/L and upon phosphorylation they can recruit phosphotyrosine phosphatases (PTPs) like SHP-1, SHP-2 or the inositolphosphatase SHIP. Recruitment of these phosphatases results in reduced phosphorylation and therefore inhibition of activation pathway effectors.

Interestingly, DAP12 contains such an ITIM sequence within its ITAM, but it is unknown if it can recruit inhibitory phosphatases [73].

A working model has been developed by Barrow et al., as an attempt to explain the polarity of DAP12-ITAM signaling: high avidity/affinity ligation of the DAP12-associated receptor leads to “strong”, dual phosphorylation of the ITAM and subsequently to cellular activation, while binding of the receptor by low avidity/affinity ligands leads to “weak” or just partial phosphorylation of the closet ITIM within the ITAM, resulting in the recruitment of inhibitory phosphatases [72-74].

4.3.3. Nasu-Hakola disease

Mutations in TREM-2 or its adaptor protein DAP12 in humans leads to a disease called Nasu-Hakola disease, also called polycystic lipomembranous osteodysplasia with sklerosing leukoencephalopathy (PLOSL). PLOSL is a recessively inherited, systemic disease and was first reported separately by Nasu and Hakola in the 1970s [75, 76]. PLOSL is a very rare, and characterized by progressive dementia and multiple cyst like lesions in the bones [77].

The clinical course of PLOSL can be divided into 4 different stages: 1) a latent, symptom free stage, 2) an osseous stage, which is characterized by pain and mild swelling of ankles and feet, and bone fractures 3) an early neurological stage, when patients show first abnormalities of the central nervous system, manifested by personality changes and behavioral alterations and, 4) a late neurological stage, when patients suffer from fully developed dementia and are usually bedridden [77]. Onset of the disease is at an age around 20 +/- 10years and the patients usually die in the 4th to 5th life decade. Time between first clinical symptoms and death can vary between 3 and 35 years; purely neurological cases without bone lesions have been reported [78].

Genetic analyses of Finnish, Japanese and Brazilian patients showed that most of patients are homozygous for mutations in the DAP12 gene, which most likely lead to the absence or at least to the production of nonfunctional DAP12. The smaller group of patients with PLOSL has a fully functional DAP12 gene, but shows homozygous

loss of function mutations in the gene encoding for the DAP12-associated receptor TREM-2. All patients with PLOSL tested so far displayed a loss of function in DAP12/TREM-2 signaling [77]. Although DAP12/TREM-2 signaling seems to have an important immunologic function, there are no reports of an abnormally higher rate of infections or neoplasias among PLOSL patients.

The fact that DAP12 is an adaptor molecule for many other receptors would assume loss of function of all DAP12-associated receptors in case of a nonfunctional DAP12. However in vitro immunological studies could not show any malfunctions of receptors other than TREM-2 [79]. These findings allow the following interesting conclusions: 1) PLOSL is caused by a loss of TREM-2 function in humans, 2) there might be compensatory mechanisms for DAP12-signaling, which can compensate for the loss of DAP12 in case of DAP12-associated receptors other than TREM-2 in human cells and 3) if there are compensatory mechanisms for the disrupted TREM-2/DAP12 signaling pathway, this mechanism fails in the brain and in the bones [77].

4.3.4. TREM-2 expression and localization

TREM-2 has been shown to be expressed on many different cells, like immature monocyte derived DCs, microglia, newly recruited peritoneal macrophages [80] and on alveolar macrophages upon induction [80, 81], bone marrow derived macrophages (BMDMs), hepatic macrophages and endothelial cells. Furthermore TREM-2 is present on a variety of cell lines: THP-1 (monocytes), U937 (pre-monocytes), CHME-5 and N9 (microglia), T98G and N2A (neuroblastoma), J774.2, IC21 and MT2 (macrophages), and RAW 264 (monocytes/macrophages) [64]. There is evidence from analysis of mRNA expression of in vitro differentiated DCs from different PLOSL patients (carrying loss of function mutations in either the TREM-2 or the DAP12 gene), that TREM-2 expression is down regulated in absence of DAP12, but DAP12 levels remain unaffected, when TREM-2 is missing [82], which is likely to be possible, since DAP12 associates also with receptors other than TREM-2.

In the mouse brain TREM-2 and DAP12 expression is detectable already in the embryo on day 17. Interestingly the transcripts, as well as the proteins show co-

localization with a microglial marker, indicating a function of TREM-2/DAP12 signaling in brain immune function [83]. Since DAP12 is a very promiscuous protein and necessary for signal transduction of many different receptors other than TREM-2, it is obvious that DAP12 has to be expressed in higher levels than TREM-2, which has been shown for murine microglia and oligodendrocytes [84]. A soluble form of TREM-2 (sTREM-2) has been found in human cerebrospinal fluid and in serum and has been shown to be upregulated in patients with multiple sclerosis (MS) or other inflammatory neurologic diseases. Foamy macrophages in actively demyelinating MS-lesions express high levels of TREM-2 [85].

Confocal studies on microglial N9 cells showed TREM-2 on the cell surface, but also TREM-2 localization in two intracellular pools, the golgi cisternae and distributed as discrete puncta in the cytoplasm [86]. A small fraction of the puncta was colocalizing with markers for the trans-golgi network (TGN) and they were just rarely colocalizing with markers for endosomes and lysosomes, so the nature of the receptor positive organelle remained unknown. Stimulation with ionomycin, a Ca^{2+} ionophore which induces large increase of the cytosolic Ca^{2+} concentration, induces an increase in the surface expression and the same is true for LPS stimulation, although the expression is increased much slower [86]. It was shown before in neurons and microglia of human and mice, as well as in human microglia and glioblastoma celllines, that TREM-2 is localized mostly within the cells, not at the surface [87]. In a mouse model for acute endotoxemia, TREM-2 expression is decreased on hepatic macrophages and endothelial cells from C3H/HeO/J mice, in contrast to TREM-1 and TREM-3, which are increased [88].

In general TREM-2 expression might be a useful marker for macrophage development and/or activation, for example it has been shown that it is expressed on infiltrating, but not resident peritoneal macrophages. Furthermore TREM-2 is the 40th most highly induced gene in human smokers [80]. Expression is up-regulated in mouse AMs after chronic 2nd hand smoke exposures [81], during allergen induced pulmonary infection and there is evidence that TREM-2 levels increase during alternative activation of AMs, by stimulation with IL-4 and IL-13. In contrast TREM-2 is down regulated in response to LPS or IFN- γ in AMs [80] and in response to IL-1 β and TNF α in mouse hepatic macrophages and endothelial cells [88].

4.3.5. Signaling and functions of TREM-2

4.3.5.1. *Immunological aspects of TREM-2 signaling*

TREM-2 is an important factor in maintenance of immune system homeostasis [89] and in contrast to its relative TREM-1, which has been shown to amplify immune responses [90], TREM-2 rather seems to be a negative regulator. Compared to wild type cells, TREM-2^{-/-} BMDMs produce more TNF- α in response to CpG, zymosan or LPS and also thioglycollate-recruited peritoneal macrophages produce more TNF- α and IL-6 in response to LPS [80]. DAP12^{-/-} BMDMs have a similar phenotype and have shown higher TNF- α production, when stimulated with TLR-ligands like CpG, DNA or Zymosan. Cytokine production could be rescued by transfecting DAP-12-deficient cells with DAP12, but a transmembrane mutant of DAP12 (TM-DAP12) failed to rescue cytokine levels, indicating that DAP12 requires the interaction with another receptor for its inhibitory function. Full rescue of cytokine levels with a TREM-2/DAP12 chimera finally showed that pairing of DAP12 with TREM-2 was required for inhibitory DAP12 signaling [71].

There is strong evidence that TREM-2 is able to form higher order complexes. TREM-2 co-precipitates with DAP12 and Plexin-A1 in COS7 cells [91]. Plexin-A1 is a semaphorin receptor which has been shown to play an important role in bone formation and immune function. Similar like TREM-2, Plexin-A1 deficiency in mice results in increased bone mass and signs of osteopetrosis because of nonfunctional osteoclasts. Furthermore Plexin-A1^{-/-} mice have DCs, which are poor in the stimulation of allogeneic T-cells and antigen specific T-cells [91] and there is other evidence that TREM-2 might play a role in the antigen uptake of DCs and in the retention of a matured stage [92].

The linker for activation of B-cells (LAB or NTAL) has been shown to be an important regulator for TREM-2 signaling, and thereby influences the inflammatory response of macrophages. LAB might suppress or antagonize TREM-2 signaling, so it is interesting that LAB deficient cells shift from IL-12p40 production to IL-10 production [93].

Syk is recruited in macrophages to ITAMs in adaptor proteins after phosphorylation and it has been shown that DAP12 requires Syk for inhibitory downstream signaling. [72]. TREM-2, DAP12 and Syk have been shown to be crucial for the fusion of macrophages to multinucleated giant cells but do not affect macrophage development. DAP12 overexpression enhances fusion of macrophages stimulated by IL4 [94].

Additionally to its regulatory role in cytokine production, TREM-2 has also been identified as a phagocytic receptor [95]. DAP12 and TREM-2 deficient BMDMs show a decreased ability for phagocytosing bacteria and TREM-2 transfected Chinese hamster ovary (CHO) cells are able to internalize *Pseudomonas aeruginosa*, *Francisella tularensis* and *Staphylococcus aureus*. The phagocytic process has been shown to be dependent on Syk as well as on Rac1 and Cdc42 [95].

In our lab experiments with TREM-2 deficient BMDMs also showed that these cells have a decreased phagocytic activity compared to wild type (WT) cells, when challenged with *E. coli* or *S. pneumoniae*. Controversially, AMs from TREM-2 deficient mice phagocytose more heat killed *E. coli* and *S. pneumoniae* than WT AMs. In an infection model of pneumococcal pneumonia with WT and TREM-2^{-/-} mice, TREM-2 deficient mice showed an improved survival (unpublished data O. Sharif).

4.3.5.2. *TREM-2 and the brain*

As mentioned before, TREM-2 and DAP12 are co-expressed in the central nervous tissue and abundant in microglia of man and mice. It is already widely accepted that TREM-2 is necessary to maintain central nervous tissue immune homeostasis, evidenced by the fact that human mutations of TREM-2 or DAP12 result in early onset dementia and that TREM-2 on microglia has been shown to be involved in phagocytosis of apoptotic material [89]. Furthermore TREM-2 is sufficient to induce phagocytosis of apoptotic neuronal cells by CHO cells, which are usually non-phagocytic [96].

Since TREM-2 is involved in the dampening of inflammatory responses, it is not surprising, that there is an involvement of TREM-2 in experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis.

In a mouse model of EAE, TREM2 is upregulated in lymphnodes and spinal cord as well as on macrophages and microglia and that blocking of TREM-2 in the effector phase of EAE, worsens the disease [97].

4.3.5.3. *TREM-2 and osteoclastogenesis*

DAP12^{-/-} and TREM-2^{-/-} monocytes, isolated from PLOSL patients show a delayed differentiation into osteoclasts [98]. Upon in vitro differentiation by stimulation with RANKL and M-CSF, the formation of polynucleated cells is strongly decreased in TREM-2^{-/-} as well as in DAP12^{-/-} cells compared to control cells from healthy donors. Furthermore, these osteoclasts have an aberrant morphology. Confocal analysis showed that these cells are smaller than control cells and show aberrant actin organization, a phenotype which was more pronounced in DAP12^{-/-} cells as compared to TREM-2^{-/-} cells. Defects in the bone resorption ability were also observed [98, 99]. Later, IL-10 was described to inhibit osteoclastogenesis by limiting TREM-2 expression on a transcriptional level and TREM-2 was found to be a regulator of RANK signaling [100].

4.3.5.1. *TREM-2 and the cytoskeleton*

TREM-2 definitely plays a role in the organization of the cytoskeleton. Although DAP12 and TREM-2 deficient monocytes (isolated from PLOSL patients with either non-functional DAP12 or TREM-2) are able to differentiate in vitro into functional DCs and to respond to microbial stimuli, these DCs show alterations in their actin cytoskeleton [82]. They have a larger surface, due to an increased number of processes, compared to wild type DCs [82]. An effect of TREM-2/DAP12 signaling was also observed in osteoclasts as mentioned before, where a staining for F-actin with phalloidin showed granules of unorganized actin filaments [99].

4.3.6. The mystery of the TREM-2 ligand

Like for almost all members of the TREM family, a ligand for TREM-2 has not been identified yet. The only exception is TLT-2, which has been shown to bind the B7 family member B7-H3 [101], an important costimulatory molecule for T-cells [102].

There is evidence for endogenous ligands on TREM-2 expressing cells. Hamerman *et al.* showed that a TREM-2/Fc chimera binds to the surface of peritoneal macrophages and BMDMs, indicating the expression of a membrane bound, endogenous ligand [72]. TREM-2-ligands have been found also on the surface of astrocytoma cells [103] and on neurons, activating a receptorcomplex upon ligation, which can be inhibited by the administration of a blocking TREM-2 antibody. The expression of these endogenous ligands was shown to be increased on neuronal and non neuronal apoptotic cells [96].

Other studies showed binding of TREM-2 to whole bacteria and to bacterial cell wall components. Experiments with a TREM2a/Ig fusion protein proved binding to a variety of gram positive and gram negative bacteria like *S. aureus*, *E. coli*, *P. mirabilis*, *S. pyogenes*. Only weak binding was observed to *P. aeruginosa* and *S. xylosus* and no binding at all to *C. albicans*. Binding of bacteria to TREM-2 transfected cells could be blocked by sTREM-2 or by adding anionic bacterial products and other anionic carbohydrates, suggesting that TREM-2 has a high affinity for anionic ligands [103].

TREM-2 was also shown to bind to lipooligosaccharide (LOS) of *Neisseria gonorrhoeae*, by far western blot and surface plasmon resonance, which also supports the hypothesis of binding preferentially anionic substances [104].

Another study identified the chaperone Hsp60 as an agonistic ligand for TREM-2 on microglia [105] and last but not least, the finding that TREM-2 associates with plexin-A1 gives evidence for TREM-2 being involved in the formation of higher order receptor complexes [91]. When searching for a TREM-2 ligand one has to take into account that it is not known if a putative TREM-2 ligand would interact with TREM-2 in such a complex or if TREM-2 would just function as orphan signaling component [61].

5. Material and Methods

5.1. Material

5.1.1. Media and supplements

<u>Antibiotics:</u>	Pen/Strep (GIBCO 15140, 10000U/ml Penicillin, 10000µg/ml Streptomycin) Puromycin (Sigma-Aldrich P-7255, 5mg/ml) Genitcin (GIBCO G-418, 50mg/ml) Blasticidin (Invitrogen R210-01, in H ₂ O, 10mg/ml) Hygromycin (Invitrogen 10687-10, 50mg/ml) Doxicyclin (Sigma-Aldrich, D9891-5G, in 70% Ethanol, 2mg/ml) Ampicillin (Sigma-Aldrich A-9518, 10mg/ml) Kanamycin (GIBCO 15165, 10000 µg/ml)
<u>Additives:</u>	FCS (fetal bovine serum, GIBCO 10082-147)
<u>RAW media:</u>	RPMI 1640 (GIBCO 21875-034) for AMs and Dulbeccos modified eagle medium DMEM (GIBCO 31885-023) for RAW cells, supplemented with 10% FCS and 1% Pen/Strep
<u>HEK media:</u>	DMEM supplemented with 10% FCS and 1% Pen/Strep, for pSelect HEK cells also with 1% Puromycin and 1% Genitcin
<u>LB media:</u>	10g NaCl, 10g Tryptone and 5g yeast extract filled up to 1L with ddH ₂ O (Antibiotics were added after autoclaving)
<u>LB agar:</u>	10g NaCl, 10g Tryptone, 5g yeast extract, 20g Agar filled up to 1L with ddH ₂ O (Antibiotics added after autoclaving)
<u>TH media:</u>	(= THB, Todd Hewitt broth) prepared by Bianca Doninger (THB: 10g Infusion from 450 g fat-free minced meet, 20g Tryptone, 2g Glucose, 2g Na ₂ CO ₃ , 2g NaCl, 0.4g Na ₂ HSO ₄ , for 1L: 36.4g THB in dissolved in ddH ₂ O and autoclaved)
<u>Blood agar:</u>	Sheep blood agar plates (Biomérieux)
<u>SOC media:</u>	2% Tryptone, 0.5% Yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl ₂ , 10mM MgSO ₄ in ddH ₂ O, 20mM sterile filtered Glucose added after autoclaving

5.1.2. Chemicals

5.1.2.1. Buffers and Solutions

Buffer	Ingredients	pH	Application
10xPBS	Morphisto (1123700500)	7.4	westernblotting, cellculture
10x TBE Buffer	109g Tris, 55g Boric acid, 4.65g EDTA in 1l ddH ₂ O (adjust to pH 8.0 with NaOH)	8.3	Preparation of Agarose gels
5x Raw detachment buffer (RdB)	15mM KAc, 150mM KCl in ddH ₂ O	n.a.*	Detaching of RAW cells, splitting
Paraformaldehyde 3.7% solution	3.7% PFA in PBS	n.a.	Fixation of cells for confocal microscopy
WCE Lysisbuffer	20mM HEPES, 500mM NaCl, 1mM EDTA, 25% Glycerol, 0.1% NP40, 1Mm DTT; 5mM NaF, 1mM Na-Vanadate, 1mM PMSF, Aprotinin (Sigma, 1:1000)	7.9	Preparation of whole cell extracts
NP-40 buffer	150mM NaCl, 1% NP-40, 50mM Tris (inhibitors like WCE buffer)	8.0	
RIPA buffer	150mM NaCl, 1% NP-40, 0.5% Na-Deoxycholate, 0.1% SDS, 50mM Tris (inhibitors like WCE buffer)	8.0	
Chaps buffer	150mM NaCl, 30mM Tris, 1% Chaps (inhibitors like WCE buffer)	8.0	
Solution C	6.06g Tris, 0.4g SDS in 100ml ddH ₂ O	6.8	Preparation of 2x SDS SB
2x SDS sample buffer (SB)	12.5ml Solution C, 5ml β -Mercaptoethanol, 10.1ml Glycerol (99%), 2g SDS, 5mg Bromphenoleblue in 50ml ddH ₂ O	n.a.	Western blotting
10x running buffer	per [I]: 30.3g Trizma base, 150g Glycine, 10g SDS	n.a.	
10x Semi-dry blotting buffer	48mM (=58g) Trizma base (Sigma; T-1258), 39mM (=29g) Glycine (Merck; 104201), 0.01% (=1g) SDS (Sigma; L-3771) in 1 ddH ₂ O;		
Blocking solution	5% BSA or 5% milk powder in PBS	n.a.	
Washing solution (PBST)	PBS with 0.1% Tween-20	n.a.	

Stripping buffer	200mM Glycine, 150mM NaCl, 0.01% Tween-20 in ddH ₂ O	2.5	
HNN-lysis buffer	50mM HEPES, 5mM EDTA, 150mM NaCl, 50mM NaF, 1mM PMSF, 1.5mM Na-Vanadate, 0.5% NP-40, protease inhibitors (Sigma P8340), 1µg/ml Avidin	8.0	Pulldown
HNN-buffer	50mM HEPES, 5mM EDTA, 150mM NaCl, 50mM NaF	8.0	

* n.a. = not adjusted

5.1.2.2. Reagents, Stimuli and Inhibitors

Substance	Company	Application
Saline	Mayrhofer Pharmazeutika	BAL
Trypanblue	Sigma-Aldrich T8154	Staining of dead cells
Ethidiumbromide.	Sigma-Aldrich 10mg/ml	DNA gels
Lidocain	GebroPharma, Xylanest 2% with Epinephrin (1:200000)	detaching cells
Bradford reagent	Biorad Protein Assay 500-0006	protein concentration
ProFluoprep	bioMerieux	Confocal microscopy
Instant milk powder	Maresi	blocking
Bovine serum albumine	Fraction V, Roche 10735094001	
Alexa-Fluor Phalloidin	Invitrogen A12379	Dyes for confocal microscopy
DAPI	Sigma 32670	
Lysotracker	Invitrogen MP07525	
Polyfect	Quiagen 301105	Tranfections
FuGene6™	Roche 11814443001	
Heat killed <i>S. pneumonia</i> (strain ATCC 6303)	prepared by O. Sharif (supplementary protocol)	Stimuli
FITC labeled <i>S. pneumoniae</i>	prepared by O. Sharif (supplementary protocol)	
LPS	Sigma	
MG132	Biomol PI 102-0005	Proteasome inhibitor
Strep Tactin sepharose	IBA, 2-1201-010	beads
Anti-HA agarose	Sigma A2095	

5.1.2.3. Kits

DNA purification kit (GE Healthcare), MiniPrep/MaxiPrep Kits (Quiagen), Quick Change - site directed mutagenesis Kit (Stratagene), TNF ELISA (R&D Duoset, EY410), IL-6 ELISA (R&D Duoset, DY406), TMB Microwell Peroxidase Substrate System (KPL, 50-76-00)

5.1.2.4. Antibodies

Primary Antibodies				
Antibody	Company	Species	Application	Dilution
anti mouse TREM-2	R&D, APC-conjugated FAB 17291A	Rat IgG 2b	FACS	1:10, 1:50
IgG isotype control	R&D, APC-conjugated, IC013A	Rat IgG 2b	FACS isotype control	1:50
anti mouse TREM-2 (G-16)	Santa Cruz Sc-22634	goat	Confocal microscopy	1:50
Anti-Myc Tag, IRDye800® conjugated	Rockland immunochemicals	rabbit	Western blotting	1:7000
Monoclonal anti-HA	Sigma H6533 1VL	mouse		1:5000
Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) mAb	Cell Signaling 9106	mouse		1:1000
Phospho-p38 MAP kinase (Thr180/Tyr182)	Cell Signaling 9211	rabbit		
Phospho-Akt (Thr308) mAb	Cell Signaling Phospho-AKT sampler kit 9916			
Phospho-GSK-3β (Ser9) mAb				
Phospho-PTEN (Ser380)				
Secondary Antibodies				
Antibody	Company	Species	Application	Dilution
anti-mouse IgG (H&L) Antibody IRDye700DX® conjugated	Rockland	rabbit	Western blotting	1:20000

anti-rabbit IgG (H&L) Antibody IRDye700DX® conjugated		goat		
IRDye 800CW anti rabbit, IRDye 680CW anti mouse	Licor	Rabbit, goat		1:15000
IgG-FITC conjugate	Millipore AP180F	Donkey anti goat	Confocal	1:400

5.1.3. Enzymes

Enzyme	Buffer	Company
Native Pfu DNA polymerase	10x Pfu buffer	Stratagene, 2.5U/ml
High Fidelity polymerase	10x HiFi buffer + 15mM MgCl ₂	Fermentas, 5U/ml
Klenow polymerase	10x NEBuffer 2	BioLabs, 5U/ml
EcoRI	10x Buffer H	Roche 11175084001
XhoI high conc.		Roche 10703770001
BamH1 fast digest	10x FD buffer	Fermentas ER0054
PstI fast digest		
Shrimp alkaline phosphatase	10x AP buffer	Fermentas EF0511
T4 DNA ligase	10x ligation buffer	Fermentas K1421

5.1.4. Primers and Vectors

5.1.4.1. Vectors

mTREM-2 pORF, hTREM-2 pORF, hTREM-1 pORF (obtained from invivogen), mDAP12 sport6, hDAP12 sport6 (obtained from imagenes), hTREM-1, pIRES (obtained from Stratagene), pDONR201, pRVNTAP GS2xT Gw, pTO HA StrepIII GW FRT (Stratagene), VSV-G was kindly provided by Prof. Superti Furga.

5.1.4.2. Primers

All primers were ordered from Sigma-Aldrich and diluted with nuclease free water up to a concentration of 100µM.

species	primer	description	Sequence 5'→ 3'	application
mouse	mP1	mTREM-2 forward	ATGGGACCTCTCCACCAGTTTCTC	<u>step 1</u> generation of overlapping TREM-2 and DAP12 fragments <u>step 3</u> P1 and P4 were also used for the amplification of the fusion product
	mP2	mTREM-2 reverse + mDAP12 overhang	AGTGTCACCTCTGGGGTGGGAAGGAG GTCTCTTGATT	
	mP3	mDAP12 forward + mTREM2 overhang	TCCTTCCACCCCAGAGTGACACTT TCCCAAGATGC	
	mP4	mDAP12 reverse	TCATCTGTAATATTGCCTCTGTGT	
human	hP1	hTREM-2 forward	ATGGAGCCTCTCCGGCTGCTCATC	
	hP2	hTREM-2 reverse + hDAP12 overhang	GCTCTGGGCCTGGGGTGGGAAGGGG ATTCTCCTTC	
	mP3	hDAP12 forward + hTREM2 overhang	CCCTTCCACCCCAGGCCAGAGCG ATTGCAGTTGC	
	mP4	hDAP12 reverse	TCATTTGTAATACGGCCTCTGTGT	
mouse	mP5	mChimera pIRES XHOI forward	GCCTCGAGATGGGACCTCTCCACCA GTTCTC	<u>Cloning</u> Introduction of restriction sites
	mP6	mChimera pIRES EcoRI reverse	GCGAATTCTCATCTGTAATATTGCC TCTGTGT	
human	hP5	hChimera pIRES XHOI forward	GCCTCGAGATGGAGCCTCTCCGGCT GCTCATC	
	hP6	hChimera pIRES EcoRI reverse	GCGAATTCTCATTTGTAATACGGCC TCTGTGT	
mouse	mP7	Mutagenesis forward	GGGATTGTTCTGGGTGCATTGGTGT TGACTCTGCTG	Site directed mutagenesis (Page purified primers)
	mP8	Mutagenesis reverse	CAGCAGAGTCAACACCAATGCACCC AGAACAATCCC	
human	hP7.1	Mutagenesis forward	GGGATCGTGATGGGAGCACTGGTGCTGACAGTGCTC	
	hP8.1	Mutagenesis reverse	CCCTAGCACTACCTCTCGTGACCACG ACTGTCACGAG	
	hP7.2	Mutagenesis forward	GATCGTGATGGGAGCACTGGTGCTGACAGTGCTC	
	hP8.2	Mutagenesis reverse	GAGCACTGTCAGCACCAGTGCTCCC ATCAGGATC	
mouse	mP9	N-Tap mTREM-2 sense attB1	GGGGACAAGTTTGTACAAAAAAGCA GGCTCCATGGGACCTCTCCACCAG	Adding att-sites for Gateway cloning
	mP10	N-Tap mTREM-2 antisense attB1	GGGGACCACTTTGTACAAGAAAGCT GGGTTCACGTACCTCCGGGTCC	
	mP11	N-Tap mDAP12 antisense attB1	GGGGACCACTTTGTACAAGAAAGCT GGGTTCATCTGTAATATTGCCT	
human	hP9	N-Tap hTREM-2 sense attB1	GGGGACAAGTTTGTACAAAAAAGCA GGCTCCATGGAGCCTCTCCGGCTG	
	hP10	N-Tap hTREM-2 antisense attB1	GGGGACCACTTTGTACAAGAAAGCT GGGTTCACGTGTCTCTCAGCCC	
	hP11	N-Tap hDAP12 antisense attB1	GGGGACCACTTTGTACAAGAAAGCT GGGTTCATTTGTAATACGGCCT	

	hP12	N-Tap hTREM-1 sense attB1	GGGGACAAGTTTGTACAAAAAGCA GGCTCCATGAGGAAGACCAGGCTC	
	hP13	N-Tap hTREM-1 antisense attB1	GGGGACCACTTTGTACAAGAAAGCT GGGTCTAGGGTACAAATGACCT	

5.1.5. Instruments

Hitachi U2000 spectrophotometer, Licor Odyssey infrared imaging system, Zeiss Confocal microscope LSM510, PCR Express Hybaid Thermocycler, Biorad UV universal hood (Gel visualization)

5.2. Methods

5.2.1. Cell culture

5.2.1.1. Alveolar Macrophages (AM)

Wildtype and TREM-2 ^{-/-} mice were euthanized with isoflurane and primary AMs were harvested by bronchoalveolar lavage (BAL). BAL fluid was centrifuged (1250rpm, 7min, RT, Rotanta 460R Hettich centrifuges) and the pellet resuspended in 1ml RAW media. Cells were counted in a counting chamber with trypanblue (1:1 dilution) to check for viability, plated in RAW media and adhered at 37°C over night. Subsequently, they were washed twice with PBS to remove erythrocytes and left to rest at 37°C at least 3h before stimulation.

5.2.1.2. RAW 264.7 cells

RAW 264.7 cells were cultured in RAW media and split three times per week at a ratio of 1:10. Cells were washed with PBS and detached by adding 1ml of 1x RAW detachment buffer (Rdb).

5.2.1.3. HEK cells

All HEK cells were split three times per week at a ratio of 1:6 by washing once with PBS and detaching using PBS supplemented with 2mM EDTA.

5.2.2. Production of a TREM-2/DAP12 fusion construct

5.2.2.1. Generation of a TREM-2/DAP12 chimera by PCR

A TREM-2/DAP12 chimeric construct consisting of the extracellular domain of TREM-2 (aminoacids 1-169) fused to the transmembrane and intracellular domain of DAP12 (aminoacids 28-114), was generated by a 3 step overlap extension PCR. The method was adapted from Wurch *et al.* [106] and this chimera was already generated before by Hamerman *et al.* and shown to be functional in dampening cytokine responses [71].

In step1, fragments with overlapping regions were generated by PCR (primers: P1, P2, P3, P4), separated and purified with a DNA purification kit after gel electrophoresis (2% agarose gel 100µg/ml Ethidiumbromide).

PCR – mix 1:

Fragment		mDAP12	mTREM2	hDAP12	hTREM2
H ₂ O	[µl]	75.3	75.3	75.3	75.3
10 x Pfu buffer		10	10	10	10
dNTPs [10µM]		2	2	2	2
Template [~2000ng/µl]		10	10	10	10
mP1 or hP1 [100µM]		-	0.4	-	0.4
mP2 or hP2 [100µM]		-	0.4	-	0.4
mP3 or hP3 [100µM]		0.4	-	0.4	-
mP4 or hP4 [100µM]		0.4	-	0.4	-
Pfu Polymerase [2.5U/ml]		2	2	2	2

PCR – program 1:

- 1) Hot start (to uncoil plasmid DNA): 3min, 94°C → hold (then primers and polymerase were added)
- 2) 10 cycles: 94°C for 30sec, touchdown from 65°C to 55°C for 45 sec, 72°C for 45sec
- 3) 25 cycles: 94°C for 30sec, 55°C for 45sec, 72°C for 45sec (1sec up, per cycle)
- 4) 72°C for 10min
- 5) Hold: 8°C o/n

The oligonucleotide primers which were used to generate the fragments were partially complementary at their 5'ends to the adjacent fragment, which means that the produced TREM-2 fragment had a 5'overhang complementary to DAP12 and the DAP12 fragment had a 5'overhang complementary to TREM-2 (Fig. 6, Fig.22).

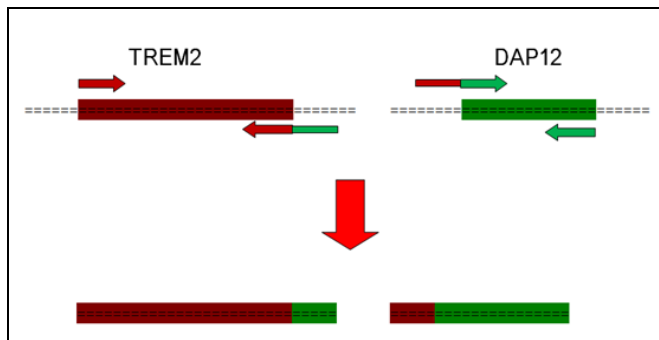


Fig.6: Scheme for the generation of TREM-2 and DAP12 fragments with overlapping regions (Step 1)

After purification and determination of the concentration the generated fragments were fused together in a 2nd PCR step, without adding primers (Fig. 7).

PCR – mix 2:

Fragment	Mouse TREM-2/DAP12 chimera				Human TREM-2/DAP12 chimera			
	mC1	mC2	mC3	mC4	hC1	hC2	hC3	hC4
H ₂ O [μl]	up to 50μl							
10 x Polymerase buffer	5	5	5	5	5	5	5	5
dNTPs [10μM]	2	1	1	1	1	2	2	1
Pfu Polymerase [2.5U/ml]	2	1	-	2	1	2	-	-
Klenow Polymerase [5U/ml]	-	-	1	-	-	-	2	1
mDAP12* [pmol]	0.5	0.5	0.2	0.2	-	-	-	-
hDAP12* [pmol]	-	-	-	-	0.5	0.2	0.2	0.2
mTREM-2* [pmol]	0.5	0.2	0.2	0.2	-	-	-	-
hTREM-2* [pmol]	-	-	-	-	0.5	0.2	0.2	0.2

* fragments generated in step 1

PCR – program 2:

25 cycles: 94°C for 30sec, 48°C for 25min, 72°C for 5min



Fig.7: Scheme for fusion of the fragments without primers (Step 2)

The 50µl PCR-mix from step 2 was then directly used as a template for the amplification of the fused construct in step 3.

PCR – program 3:

- 1) 10 cycles: 94°C for 30sec, touchdown from 65°C to 55°C for 45 sec, 72°C for 45sec
- 2) 25 cycles: 94°C for 30sec, 55°C for 45sec, 72°C for 45sec (1sec up per cycle)
- 3) 72°C for 10min
- 4) Hold: 8°C o/n

In this 3rd PCR only a forward primer for TREM-2 and a reverse primer for DAP12 were added in equimolar amounts (primers P1, P4), which means that only the fused construct can amplify. The Klenow polymerase was not working at all. Positive results were obtained with the batches with lower dNTP concentrations and higher template amounts. The obtained chimeric fragments were separated on 1.2% agarose gel (Fig. 8, Fig. 23) and purified with a DNA purification kit.

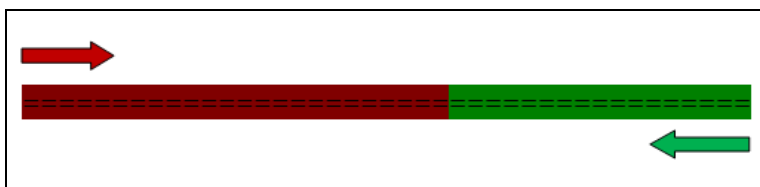


Fig.8: Scheme for the amplification of the fusion product (Step 3)

5.2.2.2. Cloning into pIRES vector

To clone the chimeric fragments into a pIRES vector, restriction sites for EcoRI and XhoI were introduced by PCR (primers: P5, P6), the fragments were separated on a 1.2% gel and purified with a DNA purification kit.

PCR – program for the introduction of restriction sites for XhoI and EcoRI:

Fragment	Mouse TREM-2/DAP12 chimera				human TREM-2/DAP12 chimera			
	mC1	mC2	mC3	mC4	hC1	hC2	hC3	hC4
H ₂ O[μl]	32.2	33.2	37.2	37.7	32.2	33.2	37.2	37.7
10 x HiFi buffer [μl]	5*	5	5*	5	5*	5	5*	5*
dNTPs (10μM) [μl]	1	0.5	1	1	1	0.5	1	1
Hi Fidelity Polymerase (5U/ml) [μl]	1	0.5	1	0.5	1	0.5	1	0.5
mP5/hP5 [μl]	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
mP6/hP6 [μl]	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Template [μl]**	10	10	5	5	10	10	5	5

* instead of the 10x Hi fidelity buffer, the 10x Pfu buffer was used to try a different Mg²⁺ concentration

** purified chimeric fragment from step 3 ($c_{mChimera} = 28.2 \text{ ng/}\mu\text{l}$, $c_{hChimera} = 23.3 \text{ ng/}\mu\text{l}$)

Empty pIRES vector and the fragments were digested with EcoRI and XhoI for 1h at 37°C in a total volume of 25μl.

	mouse	human	pIRES x 2
H ₂ O [μl]	6.25	6.25	20.45
10 x buffer H [μl]	2.5	2.5	2.5
XhoI [μl]	0.25	0.25	0.25
EcoRI [μl]	1	1	1
DNA	15	15	0.8

The vector was treated with alkalic phosphatase (AP) to avoid relegation (pIRES restriction mix + 1μl AP + 1μl H₂O + 3μl 10x AP buffer for 10min at room temperature) and purified by gel separation to remove the AP buffer. Vector and inserts were ligated at 15°C over night in a 1/2 and a 1/3 ratio (vector/insert ratio).

$$\text{ng}_{\text{insert}} (\text{for a 1:1 ratio}) = 50 (\text{ng}_{\text{vector}}) \times 0.74 (\text{kb}_{\text{insert}}) / 5.3 (\text{kb}_{\text{vector}})$$

	mouse		human	
	3:1	2:1	3:1	2:1
H ₂ O [μl]	-	1.5	2.8	4.3
T4 ligase [μl]	3	3	3	3
10x ligation buffer [μl]	1.5	1.5	1.5	1.5
Insert [μl]	8	5.8	4.5	3
pIRES	3.2	3.2	3.2	3.2

Subsequently the constructs were transformed into competent DH5α *E.coli* cells. (protocol for heat shock transformation of *E.coli* see appendix).

Transformants were inoculated in 5ml LB media (+ Kanamycin), grown over night at 37°C and plasmids were isolated by mini prep (Quiagen kit). To test for insertion in the right direction a control digest of the plasmids was performed with XhoI and BamH1.

5.2.2.3. Site directed mutagenesis

The TREM-2/DAP12 chimera transmembrane mutant (TM-mutant) was generated by exchanging the aspartic acid (D) at position +9 in the transmembrane part of DAP12, which has been shown to be required for association with other receptors [107], with alanine (A) using the Quickchange site directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. In this reaction 2 primers, which are complementary to each other and are carrying the mutation, are used for a PCR amplification of the original construct in a vector, using *PfuTurbo* DNA polymerase. The incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. After PCR the mixture is incubated with *Dpn* I endonuclease, which digests the methylated, parental DNA and thereby selects for the new, mutation-containing DNA. For the mouse fusion construct the mutagenesis worked immediately, a mutagenesis of the human fusion construct failed.

5.2.2.4. Gateway cloning

The Gateway Technology cloning system is based on the bacteriophage λ site-specific recombination system, which enables the phage to integrate into the *E.coli* chromosome and to switch between the lytic and lysogenic pathway. The recombination occurs between specific attachment (*att*) sites on the interacting DNA molecules. Upon integration of the phage, recombination occurs between *attB* sites on the *E.coli* chromosome and *attP* sites on the λ chromosome mediated by enzymes λ integrase (Int) and the integration host factor (IHF) (BP clonase). This reaction is called the BP reaction and is used by the phage for the lysogenic pathway. The BP reaction gives rise to *attL* and *attR* sites. These sites are required for the phage to excise the integrated DNA from the *E.coli* chromosome and start the lytic pathway. This reaction is called the LR reaction and is mediated by Int, IHF and the λ -phage encoded enzyme Excisionase (Xis) (LR clonase) (Fig. 9).

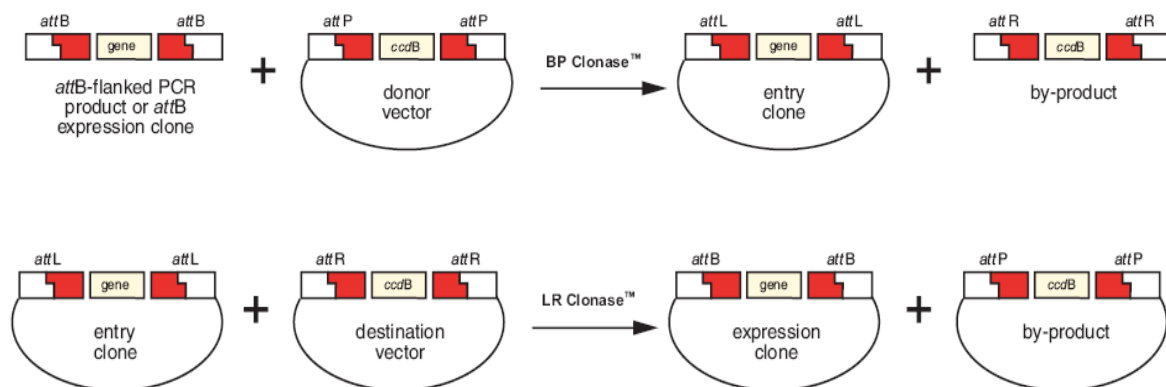


Fig.9: BP and LR reaction scheme, adapted from the Gateway Technology manual

After adding *att* sites to the constructs by PCR (primers: P9, P10, P11, see supp. Fig. 5), the fragments were cloned into a gateway compatible shuttle vector (pDONR201) with a BP reaction in order to clone the constructs into Gateway destination vectors which contain the TAP-tag (pRVNTAP GS2xT Gw for retroviral transfection of RAW cells), or the Strep-HA tag (pTO HA StrepIII GW FRT for the transfection of HEK cells), with an LR reaction.

BP reaction	
	[μ l]
H ₂ O [μ l]	up to 10
pDONR201 [μ l]	1
5x BP clonase reaction buffer [μ l]	2
<i>attB</i> containing construct (40 – 100fmol)	8
LR reaction	
	[μ l]
H ₂ O [μ l]	up to 10
Entry vector [μ l]	1
5x LR clonase reaction buffer [μ l]	2
Construct in pDONR201 [μ l]	1

5.2.3. Production of stable transfected cell lines

5.2.3.1. Viral transfection on RAW 264.7 cells

GP-293 HEK cells (packaging cell line, Clontech) were grown to a confluency of 70% and transfected with the constructs. 2 μ g VSV-G (retroviral vector) was mixed with 300 μ l serum free DMEM, 8 μ g of the pRVNTAP GS2xT Gw vector containing the construct and. 100 μ l Polyfect and the, the mixture was incubated for 10min at room temperature. Subsequently, 700 μ l of HEK media was added, the solution mixed and carefully dropped on the cells. Media was removed 12h post incubation and 6ml fresh HEK media was added and the cells were incubated for another 24h at 37°C. Target cells (RAW264.7) were seeded out in a 6 well plate at a density of 5*10⁵/well.

3 days post transfection, media was exchanged on the target cells (1ml) and they were infected with the virus. The supernatant of the HEK cells (containing the virus) was taken off (6ml), spun for 5min at 1200rpm and the HEK cells were carefully covered with another 5ml of fresh HEK media. Media was removed from the RAW cells and ~5ml of centrifuged, virus containing supernatant was added. 24h post viral infection.

RAW cells were transferred into 10cm dishes (with RdB) and a second viral transfection round was carried out on day 4, analogous to the procedure on day 3. The HEK cells were discarded and the RAW cells were expanded for FACS sorting.

5.2.3.2. *Selection for positive RAW 264.7 cells by FACS*

Before cell sorting, the cells were checked for GFP positivity cells by FACS analysis. Briefly, they were plated in a 6 well dish and cultured to 100% confluency, removed using RdB, washed twice with 2ml PBS (3% BSA), resuspended in 300µl of PBS (3% BSA) and analyzed for GFP positivity using FACS calibur (BD Biosciences).

Since 3-5% of the cells were shown to be GFP positive, the cells were sent for sorting (Peter Prinz, St. Anna Kinderspital, Vienna) and after sorting another FACS for GFP positive cells showed around 80-90% GFP positive cells.

5.2.3.3. *Transfection of Flp-InTM T-RExTM HEK 293 cells*

The procedure was based on the work of Glatter et al. [108], who described a novel integrated method for the study of protein complexes, optimizing systematic affinity purification coupled with mass spectrometry based research. Cells were grown to confluency in a 15cm plate and split 24h before transfection to 5 x 6-well plates (4ml media/well). For transfection, 6µl of Fugene6TM was added to 100µl of serum free DMEM (transfection reagent) and the mixture was incubated for 5min at room temperature. 200ng of the constructs in the destination vector were mixed with 1µg of pOG44, the transfection reagent was added, and the mixture was incubated for another 15min at room temperature before drop wise addition to the cells.

5.2.3.4. *Selection of Flp-InTM T-RExTM HEK 293 cells*

After 2 days incubation at 37°C, the cells were expanded to 10cm dishes. The next day, medium was removed and exchanged with media containing 38µg/ml Blasticidin and 260µg/ml Hygromycin to allow for selection. Media was now removed every five days to remove dead cells. The surviving cells were again cultured until they reached confluency and. detached from the plate and frozen in liquid N₂ and stored at -80°C

4.2.2.9 Pulldown experiment with Flp-InTM T-RExTM HEK 293 cells

Cells were expanded to 5x 15cm plates, grown to 70-80% confluency and expression induced by addition of 1µg/ml Doxycyclin. 24h post Doxycyclin treatment, each plate was washed with 10ml cold PBS (supplemented with 0.1mM MgCl₂ and 0.1mM CaCl₂) and the cells were harvested with PBS containing 1mM EDTA, centrifuged, snap frozen in liquid N₂ and stored at -80°C.

The frozen pellet was resuspended in 5ml HNN-lysis buffer (1ml/dish) with Avidin, incubated on ice for 10min and spun down (13K, 20min, 4°C). During centrifugation Strep Tactin sepharose beads were equilibrated, by loading 400µl slurry on BioRad Biospin columns (#732-6008) and washing twice with 1ml HNN-lysis buffer without Avidin.

Before purification an aliquot of the lysate was taken for western blotting.

For the first purification step the lysate was slowly added to the beads (lysate entered the resin by gravity flow). The beads were then washed 3x with HNN-Lysis buffer and elution was performed by adding 3x 200µl and 1x 300µl HNN-buffer with supplements, containing 2.5mM Biotin.

For the second purification step, 100µl anti-HA beads (Anti-HA Agarose, Sigma A2095) (200µl slurry)/ sample were washed 1x in HNN-Lysis buffer, spun down for 1min at 1000rpm and resuspended in HNN-Lysis buffer. The beads were then added to the eluate from the first purification and incubated for 1h on a rotating shaker at 4°C. The suspension was transferred into a new column, the beads were washed 3x with 1ml HNN-lysis buffer (with NP40 and inhibitors) and 2x with 1ml HNN buffer (without NP40 and inhibitors). Then elution was done by adding 5x 100µl 0.2M Formic acid, pH 2.5 and the eluate was neutralized by adding 160µl of 1M NH₄CO₃ (pH 8.8). Before the samples were flash frozen in vials for mass spectroscopy, another aliquot was taken for western blotting.

5.2.4. Stimulations

All stimulations for western blotting were performed in serum free media. All others (ELISA, Confocal microscopy) were carried out in presence of 10% FCS.

HEK cells were stimulated with heat killed *S. pneumoniae*. at a concentration of 2×10^8 CFU/ml, RAW cells and alveolar macrophages with a concentration of 2×10^7 CFU/ml. LPS from *E.coli* was used as a control at a concentration of 1 µg/ml.

5.2.5. Killing assay

S. pneumoniae was grown according to supplementary protocol (appendix). Cells were plated in a 24 well-dish at a density of 2×10^5 /well in RPMI with 10% FCS (no antibiotics), left in the incubator for 3h to adhere, washed 2x with PBS and covered with 500 µl fresh media. *S. pneumoniae* was added to the wells (MOI 100) and spun on to the cells by centrifugation (2000rpm, 5min, 4°C). Cells were incubated at 37°C for 10min to allow phagocytosis and subsequently all wells were washed five times with ice cold PBS to remove extracellular bacteria. Cells from 3 wells were immediately lysed after washing in 1ml ddH₂O (timepoint 0). The other cells were covered with 500 µl fresh media and incubated at 37°C. 5, 10, 30 and 60min post incubation, another 3 wells were washed three times each with ice cold PBS and lysed in 1ml of ddH₂O. The lysates were then diluted 1:10 in saline up to a dilution of 10^{-3} and 50 µl of each dilution was dropped on blood agar plates. CFUs were counted on the next day.

5.2.6. Enzyme-linked Immunosorbent Assay (ELISA)

Retroviral transfected RAW cells were plated in a 96-well dish with a density of 4×10^5 /well and stimulated the next day with heat killed *S. pneumoniae* (2×10^7 CFU/ml) for 6h and 20h. TNFα and IL-6 levels were in the supernatant using ELISA (R&D). Samples were diluted in ELISA washing buffer with a ratio of 1:2 for the 6h

stimulation and 1:5 or 1:10 for the 22h stimulation. Fluorescence activated cell sorting (FACS)

2×10^5 cells per sample in 100 μ l of FACS buffer (PBS containing 3% BSA) was incubated for 20 min in the dark with either anti TREM-2 antibody (1:50 or 1:10 dilution) or isotype control antibody (1:50 dilution). Subsequently, cells were washed with 1ml FACS-buffer centrifuged (1250rpm, 5 min), supernatant removed and TREM-2 surface expression measured by FACS analysis.

5.2.7. Confocal microscopy

Cells were plated at a density of $1-3 \times 10^5$ /well in 8-well chamberslides (Lab-Tek Chamberslide system) and incubated over night at 37°C. All incubation steps were performed at room temperature except the incubation with the primary anti-TREM-2 antibody, which was carried out at 4°C

Cells were washed twice with 200 μ l PBS and fixed by covering them with a solution of 3.7%PFA for 10min. Subsequently, PFA was removed by washing twice with PBS and cells were blocked using 1%BSA in PBS for 0.5h.

For the TREM-2 staining the cells were blocked with PBS with 10% serum (mouse and rabbit were tried) and 1% BSA for 0.5h. For the TREM-2 staining, cells were after blocking incubated over night with anti TREM-2 antibody diluted 1:50 in PBS for the surface staining and in PBS with 0.1% Triton-X for the intracellular staining over night at 4°C. After three times washing with PBS, cells were incubated with the secondary FITC labeled antibody diluted 1:400 in PBS for the surface staining and in PBS with 0.1% Triton-X for intracellular staining for 30min. From this time on, the cells were protected from light. After the staining the cells were again washed three times with PBS. For the actin staining, Alexa Fluor-conjugated phalloidin was diluted from the stock (6.6 μ M) 1:600 in PBS with 1% BSA and added on the cells (after blocking and washing) and incubated in the dark for 20-30min, followed by two washing steps with PBS. Visualization of the nucleus was achieved by covering the cells with a DAPI solution in PBS (1 μ g/ml) and incubated for 10min in the dark.

For the phagocytosis experiment, cells were stimulated before fixation with FITC-labeled heat killed *S. pneumoniae* with an MOI of 100 for 1h, together with LysoTracker (60nM), which marks lysosomes because of their low pH. After stimulation cells were fixed and the nucleus stained with DAPI as described above.

After the last washing step, the cover of the chamber slide was removed and shortly air dried. Cells were covered with a coverslide (24 x 50mm, VWR) using mounting media (Fluoprep). Slides were visualized via confocal microscopy (Zeiss LSM 510).

5.2.8. Preparation of cell extracts

All extracts which were prepared without denaturing conditions were made on ice, freshly prepared buffers and tubes were pre-chilled.

Macrophages were washed with PBS, covered with WCE buffer and left on ice for a couple of minutes, scratched off from the well and transferred into eppendorf tubes. HEK cells which were easy to detach were directly transferred in eppendorf tubes and covered with WCE lysis buffer. The amount of lysis-buffer was dependent on the amount of cells (5×10^5 – 5×10^6), ranging from 75 μ l to 200 μ l. Cells were passed through a 25G needle ten times, left on ice for 30min, spun at 14000rpm, 4°C, 10min and supernatant was transferred into a fresh pre-chilled eppendorf tube, frozen in liquid N₂ and stored at -80°C. The protein concentration was measured by Bradford.

Because of the huge amount of cells which are required for whole cell extracts, cells were also lysed directly in hot (95°C) SDS-sample buffer, if the samples were used for western blotting. The disadvantage of this method is, that it is not possible to determine the protein concentration, so it is necessary to precisely plate equal amounts of cells and use equal amounts of lysis-buffer.

5.2.9. SDS-Page

Samples which were directly lysed in SDS-sample buffer were boiled for 5min at 95°C and directly used, without determining the protein concentration. 25µg of protein were loaded from samples which have been lysed in WCE lysis buffer. The samples were 1:1 diluted with 2x SDS-sample buffer and boiled for 5min at 95°C prior to gel loading. Samples were loaded to a 10% or 12% SDS-page gel and electrophoresis was performed for 1h at 120V (Biorad MiniProtean electrophoresis System).

5.2.10. Western blot analysis

1x semi-dry blotting buffer was prepared by dilution of the 10 x stock 1 in 10, with ddH₂O and adding 20% Methanol (Sigma; M1770). Proteins were blotted onto a PVDF membrane (Biorad, Immun-blot PVDF membrane, 0.45µm poresize) using semi-dry transfer for 1h at 10-12V (Biorad Transblot SD semidry transfer cell). Membranes were blocked with either PBS containing either 5% BSA or 5% milk powder for 1h at room temperature. If milk was used for blocking, the membranes were washed twice with PBST (0.1% Tween20) before incubation with the primary antibody.

The primary antibodies were used in a dilution of 1:1000 in PBST with 2% BSA and incubated over night on a shaker at 4°C. If the signal was very weak the membrane was incubated with the primary antibody on the next day for another 1-2h at room temperature. Subsequently, membranes were washed three times 15min with PBST and incubated with secondary, fluorescently labeled antibody in PBST with 2% BSA at a concentration of 1:20000 (Rockland) or 1:15000 (Licor). The membrane was washed three times for 15min with PBST, to reduce the background in the last washing step 0,01% SDS was added to the washing buffer. Detection was performed using Licor Odyssey infrared imaging System. .

6. Results

6.1. TREM-2 and phagocytosis

6.1.1. Enhanced phagocytosis by TREM-2 ^{-/-} AMs

As mentioned before, TREM-2 deficient AM have been shown to exert an increased phagocytic activity in a FACS based phagocytosis assay (Fig. 10), when stimulated with FITC-labeled *S. pneumoniae* or *E.coli* (O. Sharif, unpublished data).

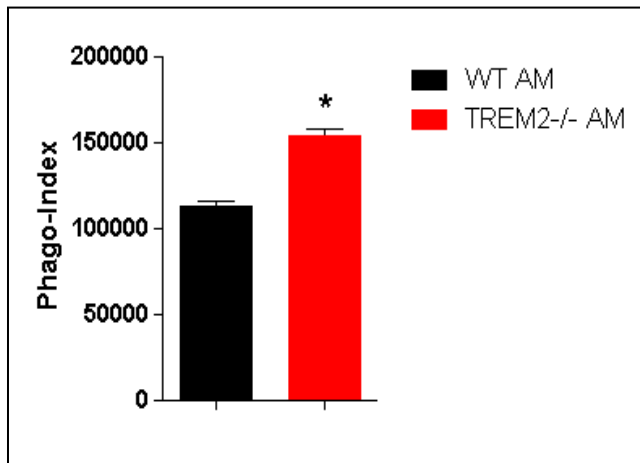


Fig.10: FACS based phagocytosis assay after stimulation of WT and TREM-2^{-/-} AMs with FITC-labeled *S. pneumoniae* (O. Sharif unpublished data)

Since this result was very surprising and is in conflict with previous publications, which identified TREM-2 as a phagocytic receptor [95], the idea was to verify this result using another method. Therefore, confocal microscopy on AMs from WT and TREM2^{-/-} mice incubated with FITC labeled bacteria was conducted.

Fig.11 and 12 shows WT (left) and TREM-2^{-/-} (right) AMs with ingested FITC-labeled *S. pneumoniae*. (green). Nuclei are visualized in blue, in red lysosomes are seen, stained with LysoTracker. Depending on the intensity of the LysoTracker staining the color of the bacteria ranges from yellow to green.

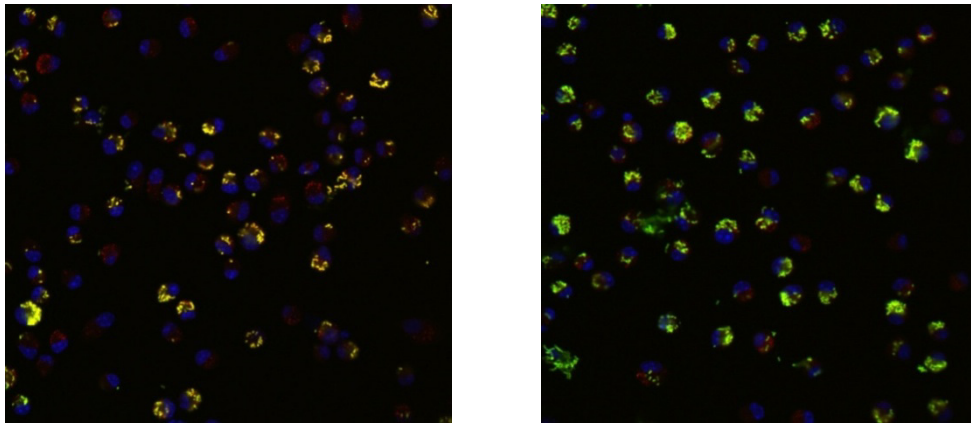


Fig.11: Confocal microscope picture of WT and TREM-2^{-/-} AMs with ingested FITC-labeled bacteria

Cells	positive	negative	total	% positive
WT AMs	54	26	80	67,50
	11	12	23	47,83
	60	74	134	44,78
	48	58	106	45,28
TREM-2 ^{-/-} AMs	57	20	77	74,03
	53	29	82	64,63
	56	15	71	78,87
	37	14	51	72,55

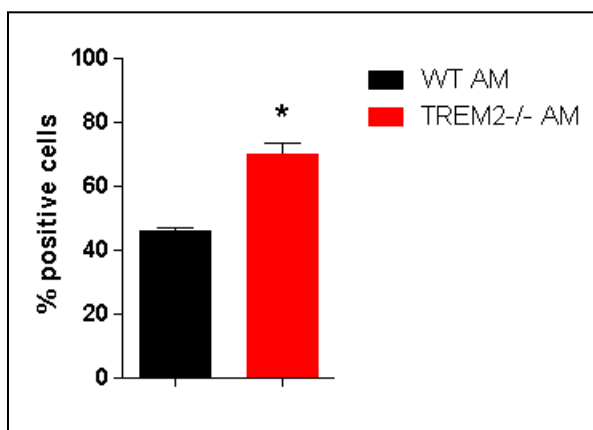
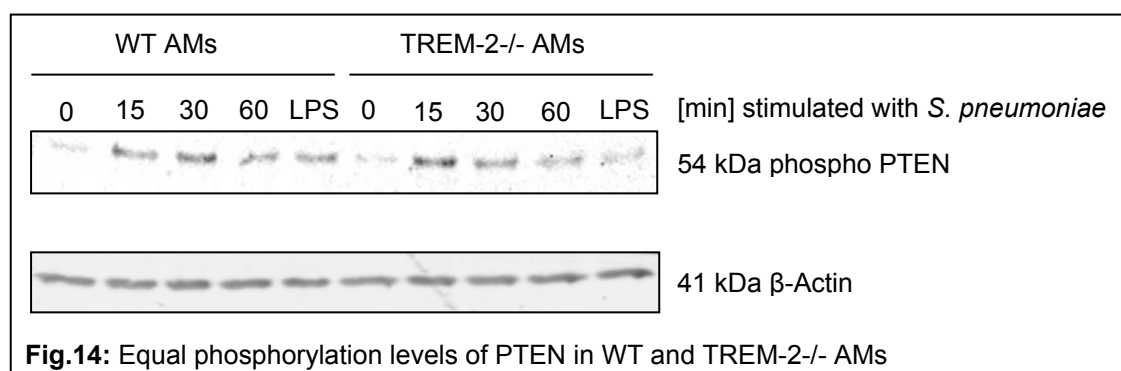
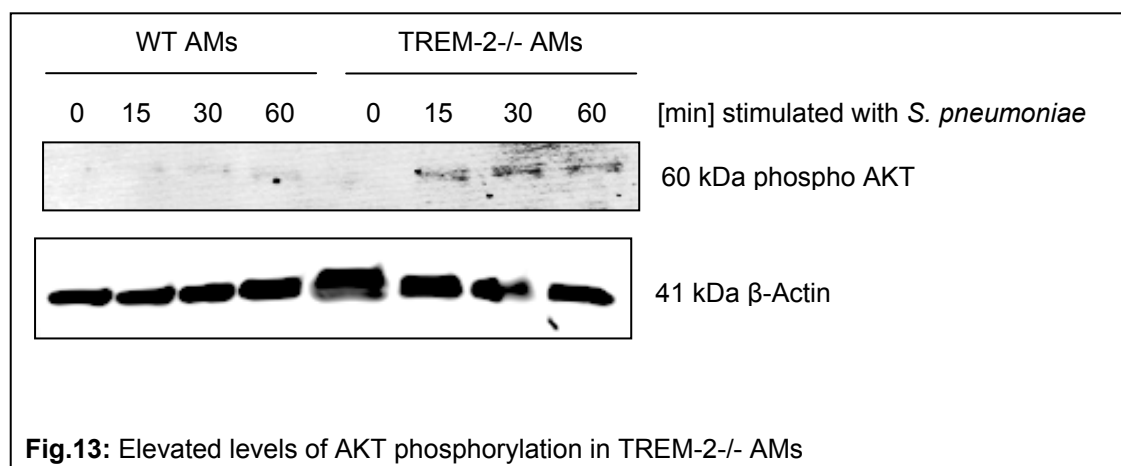


Fig.12: Calculation of the percentage of cells which were phagocytosing bacteria

Four squares were counted and the percentage of positive cells was calculated (Fig. 12). The amount of ingested bacteria was not considered. Every cell containing any bacteria was counted as positive. From this result the conclusion was that TREM-2 deficient AMs indeed phagocytose more *S. pneumoniae* compared to wildtype cells.

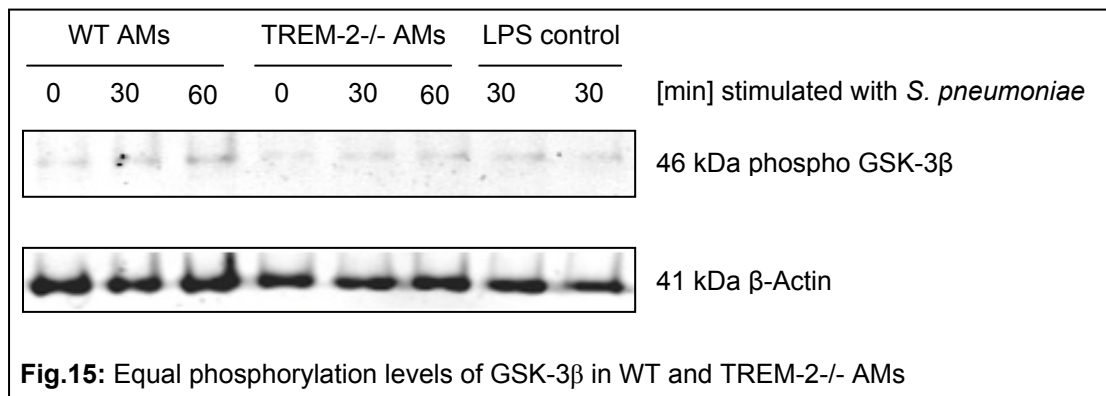
6.1.2. Elevated PI3 Kinase activity in TREM-2 ^{-/-} AMs

After verifying that TREM-2 deficient AMs have a higher phagocytic activity, when challenged with *S. pneumoniae*, the question evolved which mechanism was responsible for this increased uptake. PI3-Kinase has been shown to be involved in the regulation of cytoskeletal reorganization during phagocytosis [57]. AKT is an important target of PI3-Kinase. When stimulated with *S. pneumoniae*, TREM-2^{-/-} AMs show higher levels of phosphorylated AKT (Thr308) (Fig. 13), whereas levels of the phosphorylated PI3-Kinase antagonizing phosphatase PTEN (Ser380) did not differ between genotypes (Fig. 14), indicating equal activity of PTEN.



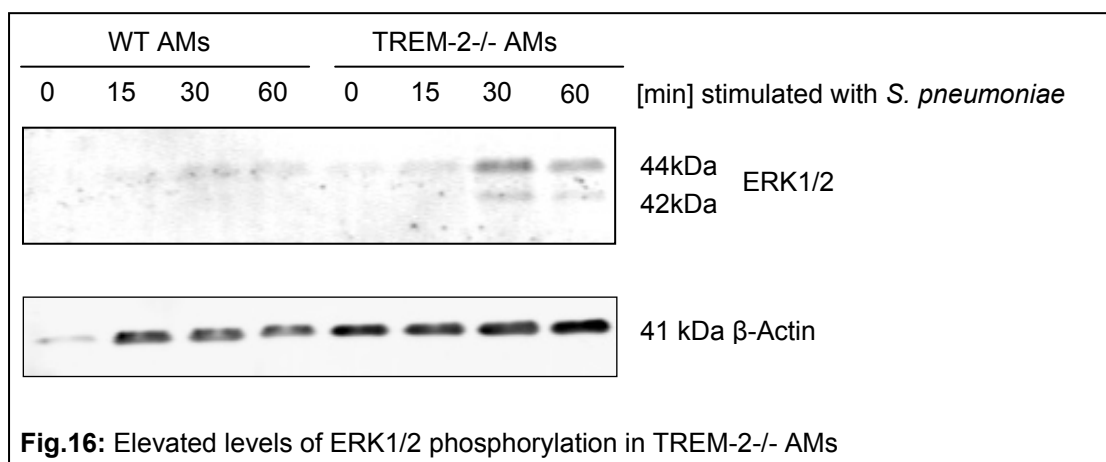
A FACS based phagocytosis assay showed that treatment of the cells with the PI3-kinase inhibitor wortmannin equalizes the phagocytosis of WT and TREM-2^{-/-} AMs (unpublished data, O. Sharif), suggesting that enhanced PI3-kinase in TREM-2 deficient cells might be responsible for the enhanced phagocytic activity.

Surprisingly also the levels of phosphorylated glycogen synthase kinase-3 β (GSK-3 β) remained unaffected (Fig. 15), which is an important substrate of activated AKT. AKT phosphorylates and thereby inactivates GSK-3 β . It has been published that active GSK-3 β is important in the regulation of TLR-mediated cytokine production [109].



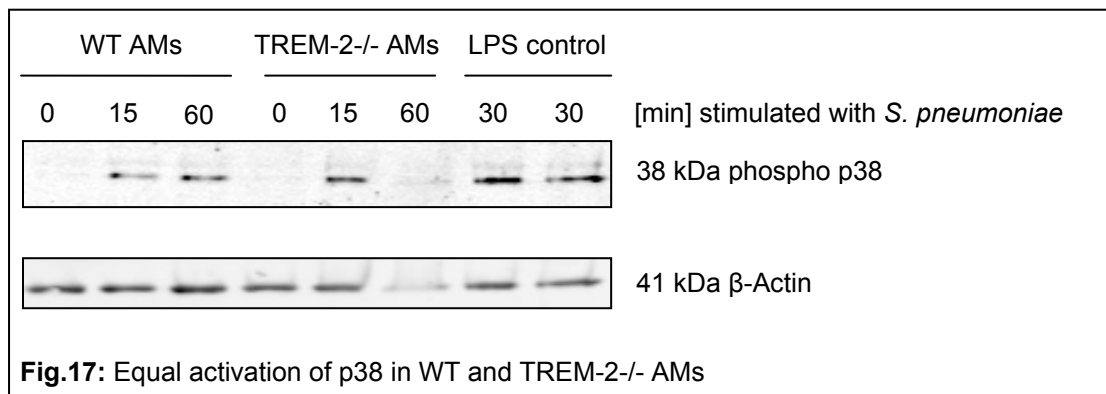
6.1.3. More ERK-phosphorylation in TREM-2^{-/-} AMs

It has been shown that there is enhanced phosphorylation of the MAP kinase ERK in DAP12 deficient bone marrow derived macrophages [110]. Therefore, it is likely that TREM-2 deficiency may impact this MAP-kinase. Indeed, upon stimulation with *S. pneumoniae*, TREM-2^{-/-} AMs show higher levels of ERK1/2 phosphorylation compared to WT AMs (Fig. 16).



Although, there was not enough protein loaded for the unstimulated WT sample, this result was observed in another independent experiment, but the differences between WT and TREM-2^{-/-} AMs was smaller (data not shown). Nonetheless, in both experiments highest levels of phospho-ERK were seen after 30min stimulation and then decreased again (Fig 16).

Phosphorylation of the MAP-kinase p38 seemed to be unaffected at least at 15 min (Fig. 17), which is a phenotype similar to DAP12^{-/-} BMDMs stimulated with LPS [110]. It is difficult to conclude something from the 60 min time point due to insufficient protein amounts in the TREM2^{-/-} 60 min sample.



6.1.4. Analysis of the actin cytoskeleton by confocal microscopy

Since it has been published that TREM-2 deficiency leads to aberrant organization of the actin cytoskeleton in DCs and in osteoclasts [82, 99], filamentous actin (F-actin) of WT and TREM-2^{-/-} AMs was stained with Alexa Fluor-coupled phalloidin and visualized by confocal microscopy to determine whether TREM2 deficiency has a similar effect in alveolar macrophages (Fig. 18).

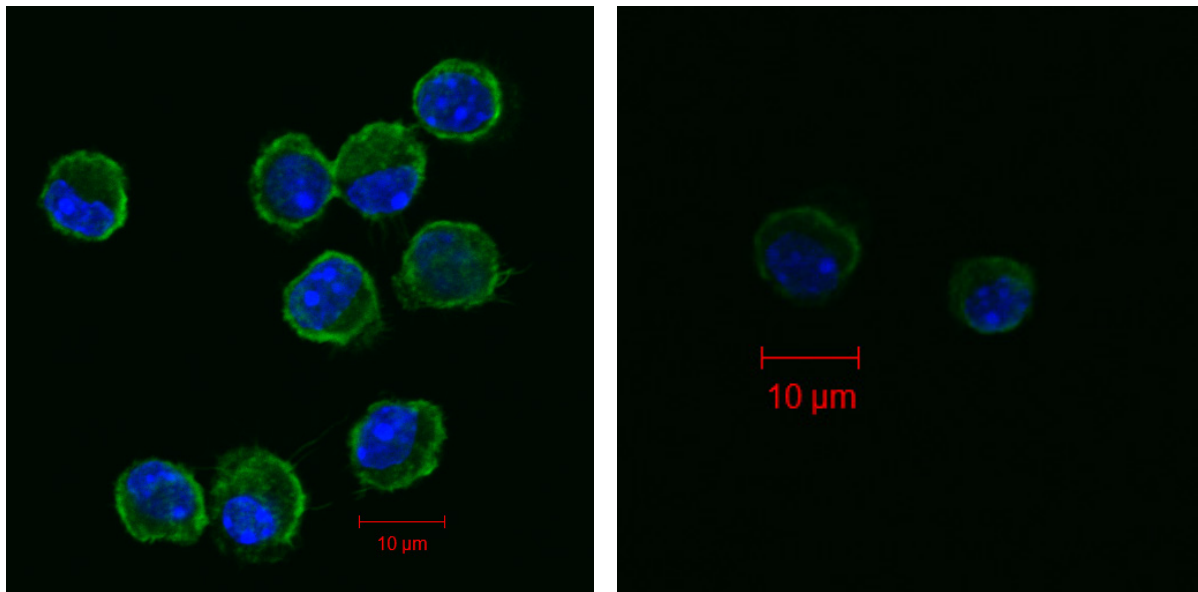


Fig.18: Staining of F-actin with Alexa Fluor-conjugated phalloidin of WT AMs (left) and TREM-2^{-/-} AMs (right)

Both cell types, WT and TREM-2^{-/-} AMs, have a round shape and show no visible differences in the amount of protrusions or cell size (~ 10μm). Furthermore, the distribution of F-actin seems so be the same, but interestingly the signal intensity of TREM-2 deficient AMs was lower. This result was obtained in two independent experiments, when WT and TREM-2^{-/-} cells were visualized with the same settings.

6.1.5. TREM-2 has no effects on the killing properties of AM

Since TREM-2^{-/-} AMs show enhanced phagocytosis, the question was if these cells also show differences in their ability to kill ingested bacteria. To answer this question a killing assay was performed to compare the killing properties of WT and TREM-2^{-/-} AM and showed that TREM-2 deficiency does not affect the killing of ingested *S. pneumoniae* (Fig. 19).

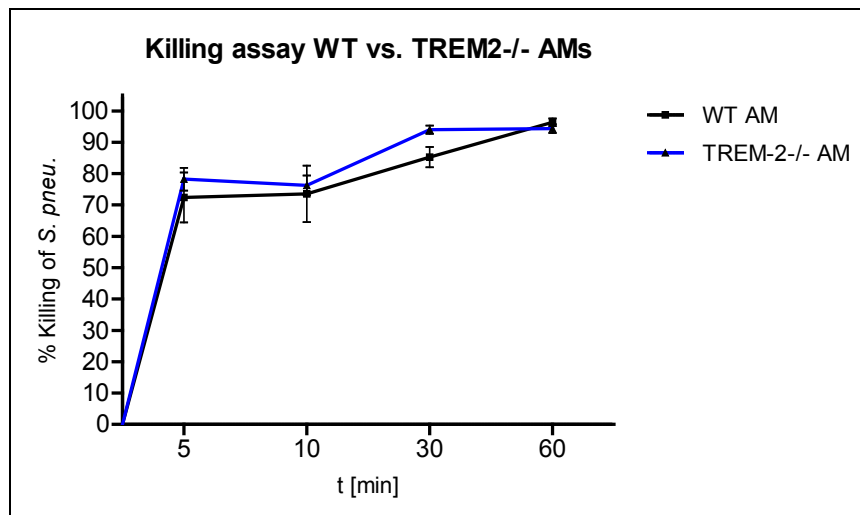


Fig.19: Killing curve of *S. pneumoniae*. of WT vs. TREM-2^{-/-} AMs

6.2. TREM-2 staining

It has been previously observed that TREM-2 is located intracellular in the Golgi apparatus of microglia [86]. Therefore, we wanted to determine whether it was intracellularly located in AMs, but it turned out to be difficult because of the lack of good antibodies. The secondary antibody caused a very strong unspecific signal on cells which were not pretreated with the primary anti TREM-2 antibody. Blocking with 1% BSA and 10% mouse serum finally worked, but still needs to be optimized.

Intracellular staining for TREM-2 showed TREM-2 to be localized in small aggregates, distributed within the whole cell (Fig. 20), similar to the “puncta” which were observed by Prada et al. in microglia N9 cells. These puncta were also observed extracellular in cells which were stained with and without triton-X. The

surface staining looked different and showed a more homogenous distribution of TREM-2 than in the cells which were treated with detergent (Fig. 20), indicating that TREM-2 is homogenously expressed on the cell surface. The intracellular staining might give evidence for the intracellular localization of TREM-2, but needs to be repeated with another detergent.

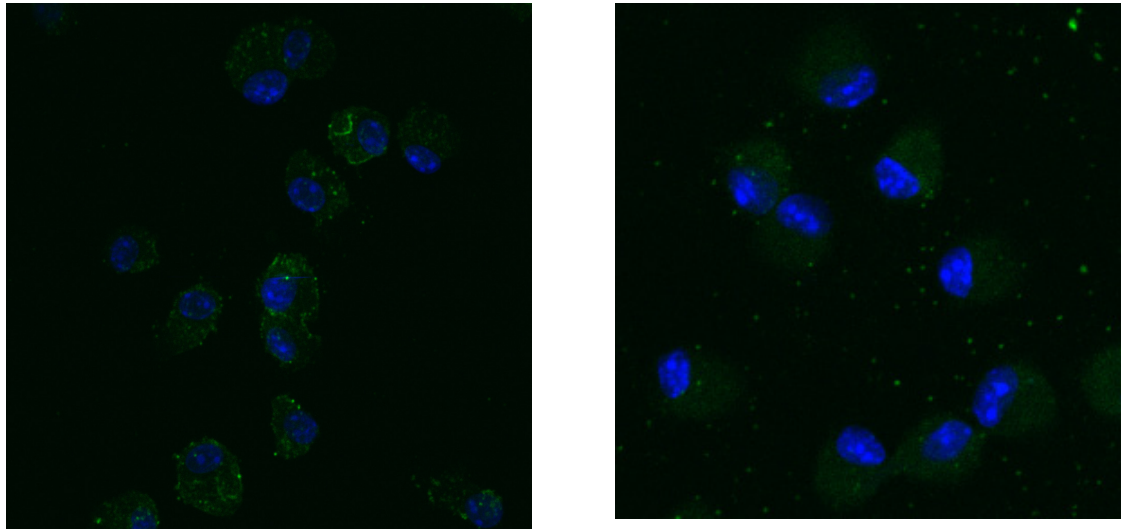


Fig.20: Staining of TREM-2 in WT AMs: intracellular (left) and surface (right)

Controls with the secondary antibody without pre-treating the cells with the primary antibody still gave a weak signal (Fig. 21), even after blocking with serum, but luckily there was a significant difference in the intensity between the TREM-2 stained cells and the unspecific signal from the secondary antibody.

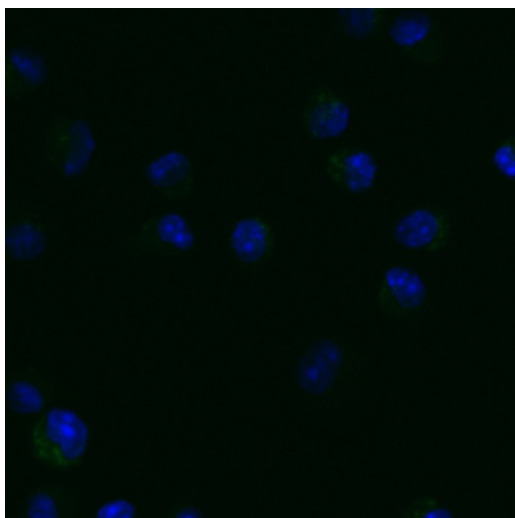
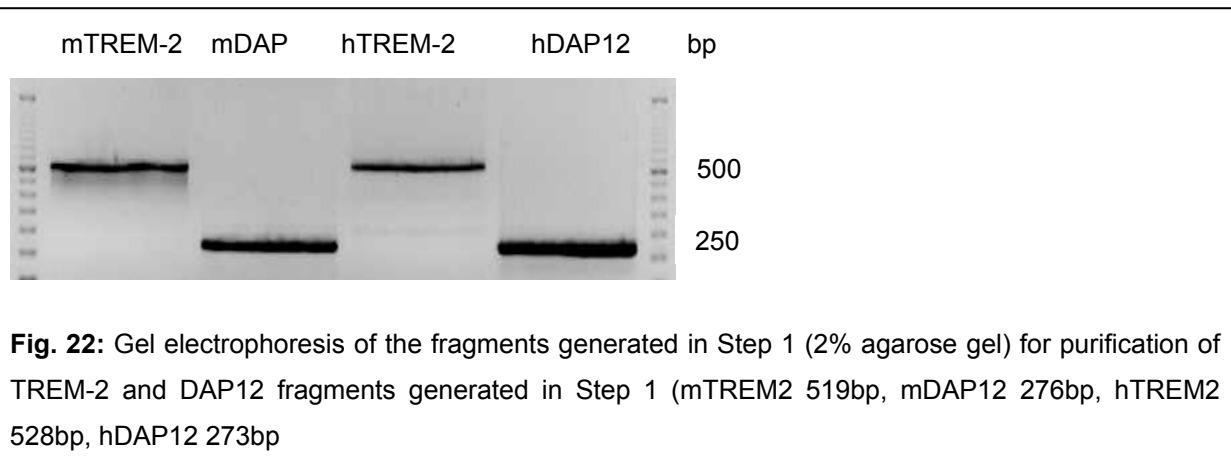


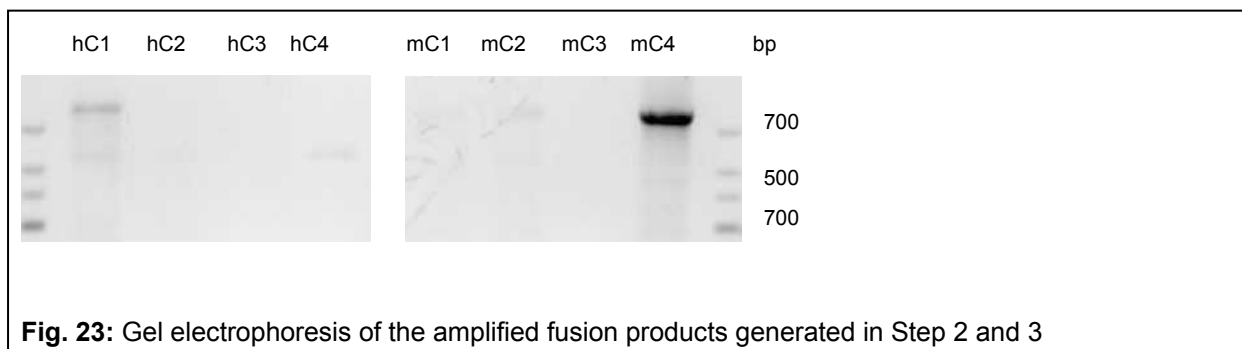
Fig.21: Staining of WT AMs stained only with the secondary antibody in a dilution of 1:400

6.3. Generation of a TREM-2/DAP12 chimeric construct

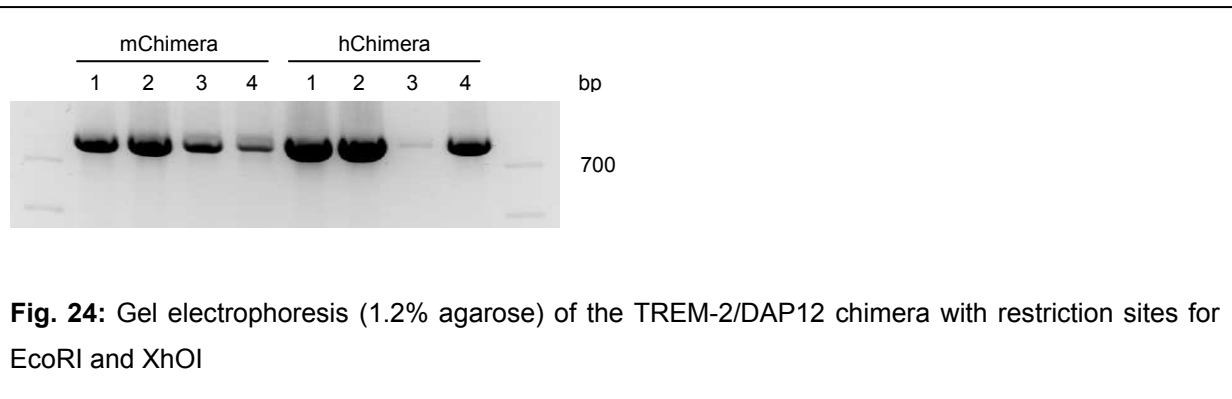
The production of TREM-2/DAP12 chimeric constructs for transfection into RAW cells (mouse) and for transfection into HEK cells (human) was successful after couple of trials. The production of overlapping fragments or TREM-2 and DAP12 (mouse and human) is seen in Fig. 22 and worked well.



Especially the second PCR step, when the fusion of the TREM-2 and DAP12 should take place turned out to be a highly sensitive process, which doesn't work anymore when the dNTP concentration is too high or the template concentration too low. After the third PCR step DNA fragments with the expected size of the chimeric molecule (hChimera 777bp, mChimera 771 bp) were obtained (Fig. 23)

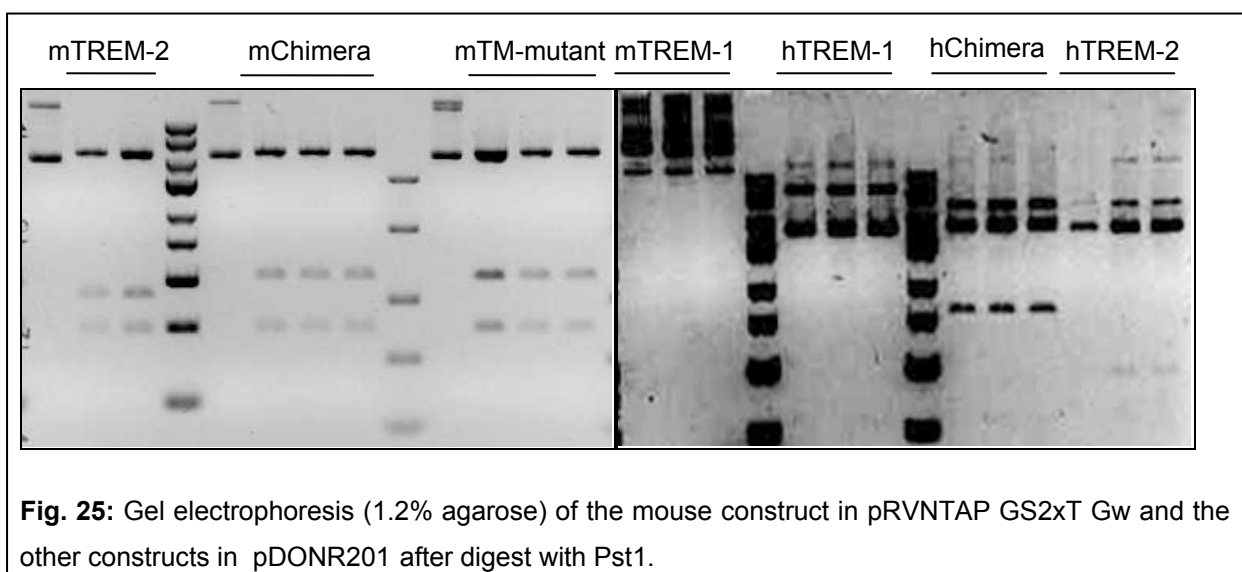


By PCR the fusion constructs were amplified and restriction sites for cloning into pIRES were introduced (Fig. 24).



After cloning into the pIRES vector, a control digest with BamHI showed all clones to have the fragment inserted in the right direction. The fragment in pIRES was used for the production of a TM-mutant chimera by site directed mutagenesis, which was successful.

All constructs were cloned into the gateway donor vector pDONR201 and a control digest with PstI was performed (Fig. 25). Subsequently the constructs were cloned into the corresponding destination vectors, hTREM-1 and hTREM-2 into pTO HA StrepIII GW FRT, mTREM-2, mTREM-1, mTREM-2/DAP12 chimera and the TM-mutant chimera into pRVNTAP GS2xT Gw. mTREM-1 was ordered in a gateway compatible donor vector and a PstI control digest was performed after the LR reaction (Fig.25).



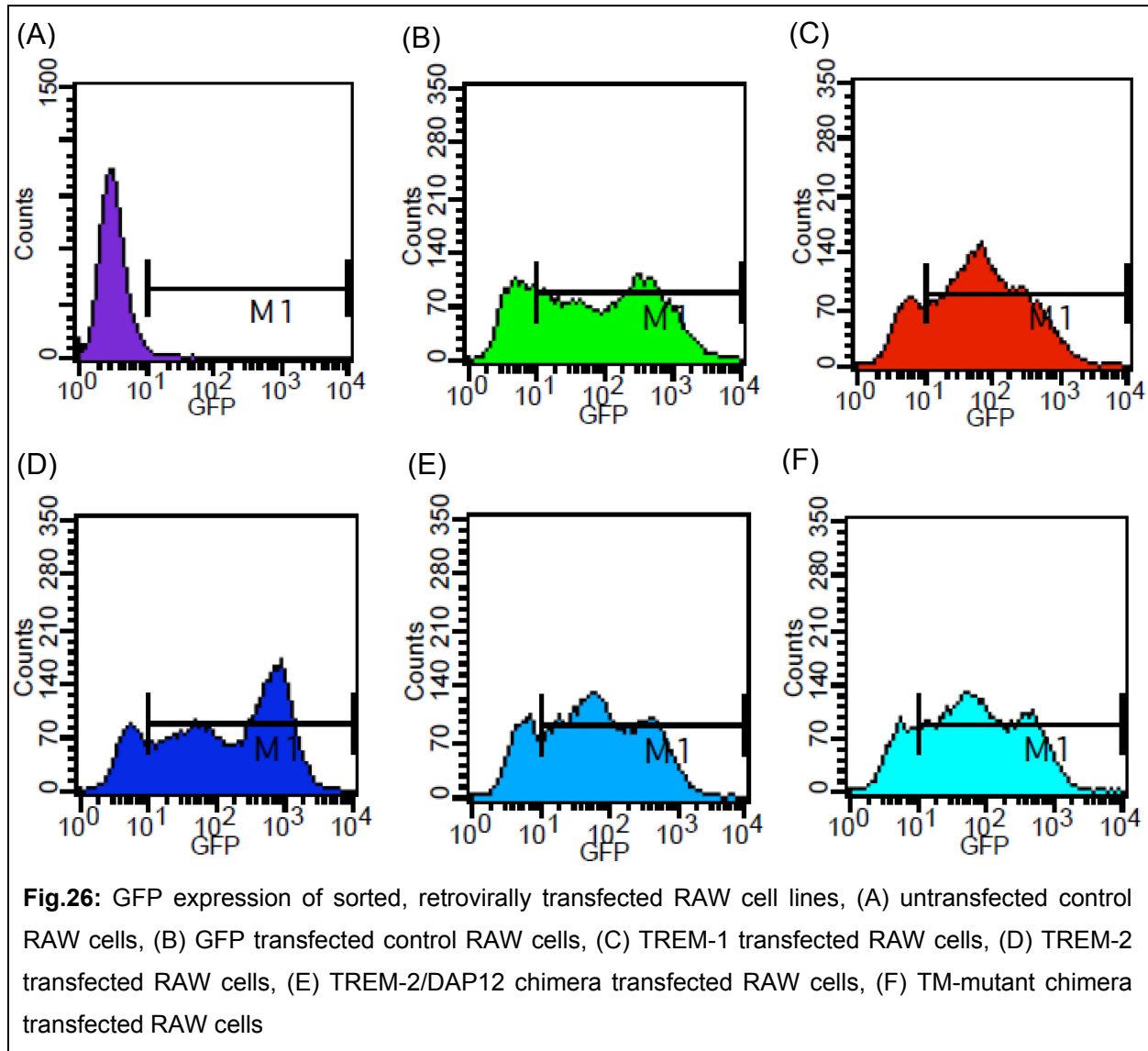
All constructs showed to be positive and were sent for sequencing. Unfortunately the human TREM-2/DAP12 chimera showed to have a point mutation in the TREM-2/DAP12 overlapping region (sequencing results not shown) and was therefore not used for the production of stable transfected HEK cells.

6.4. Stable transfected cell lines

RAW 264.7 cells transfected with GFP, TREM-1, TREM-2, TREM-2/DAP12 chimera or the TM-mutant chimera were generated, as well as Flp-InTM T-RExTM HEK 293 cells carrying TREM-2 and TREM-1 with a Doxycyclin inducible promoter. In both cases the transfection was successful, but the constructs failed to be expressed by the cells on protein level.

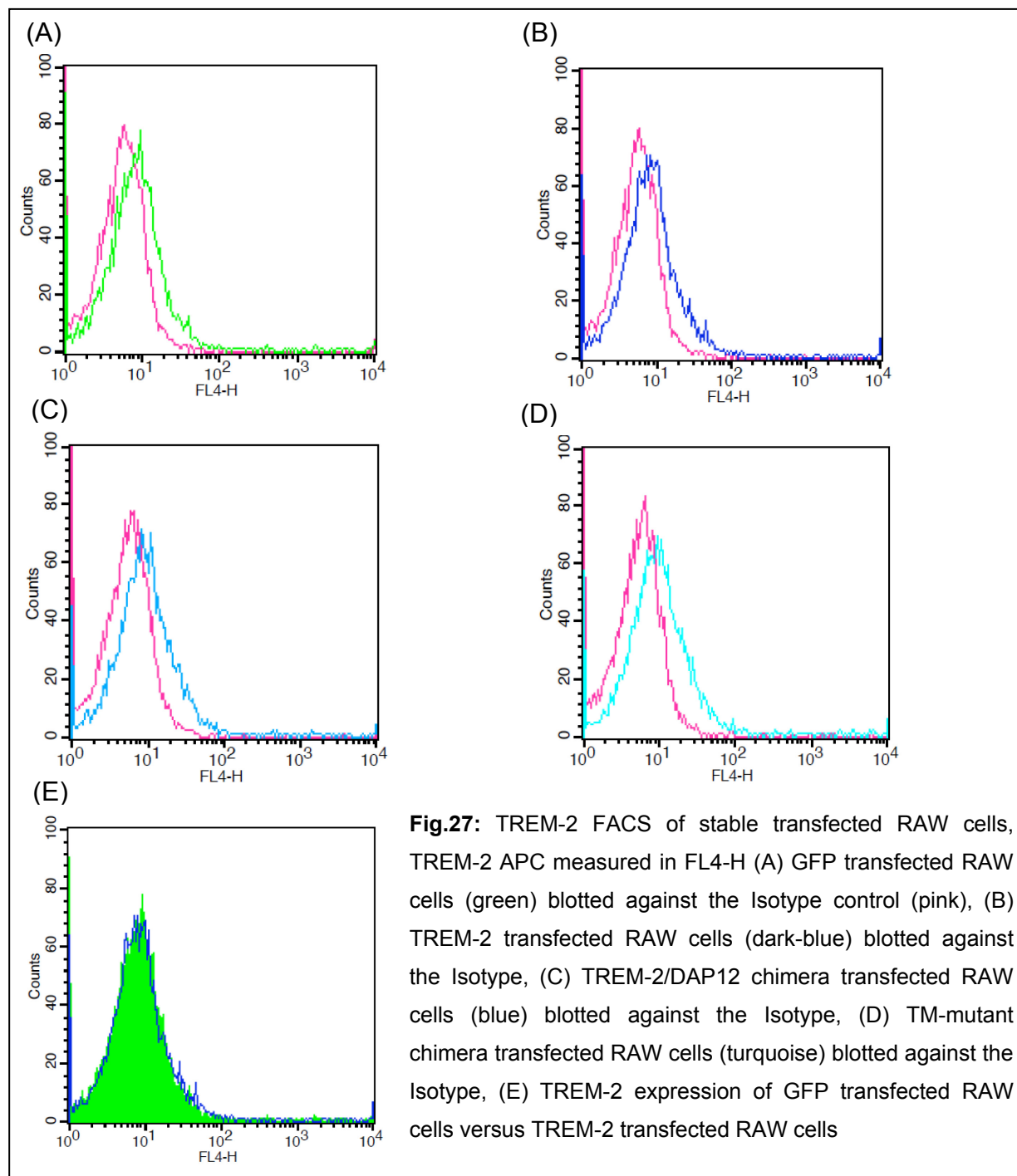
6.4.1. FACS of transfected RAW 264.7 cell-lines

A selection for transfected RAW cells was done by sorting the cells for GFP positive cells. Before cell sorting, ~5% of transfected RAW cells showed expression of GFP, indicating that they were successfully transfected with the construct. After sorting 80-90% of the cells expressed GFP (Fig. 26)



Although the cells showed GFP positivity indicating successful transfection, neither the TREM-2 transfected cell line nor the cell lines transfected with the fusion constructs showed overexpression of TREM-2, when the cells were stained with a

TREM-2 antibody. Low levels of endogenous TREM-2 surface expression were detectable (Fig. 27, A-D), although the antibody should be used in a higher concentration. Importantly, TREM-2 expression was not elevated in the TREM-2 transfected RAW cells compared to the GFP-transfected control RAW cells (Fig. 27, E). The conclusion was that the constructs were most likely not expressed well or might get dislocalized and degraded.



An intracellular FACS for TREM-2 was performed but failed to detect any TREM-2 neither intracellular nor on the surface, indicating that the staining was not working well. The experiment was not repeated due to the result shown on Fig. 29.

6.4.2. Enhanced levels of IL-6 in transfected RAW cells

To check functional effects of overexpression of all the constructs, cytokine production was measured after stimulating the cells with *S. pneumoniae*.

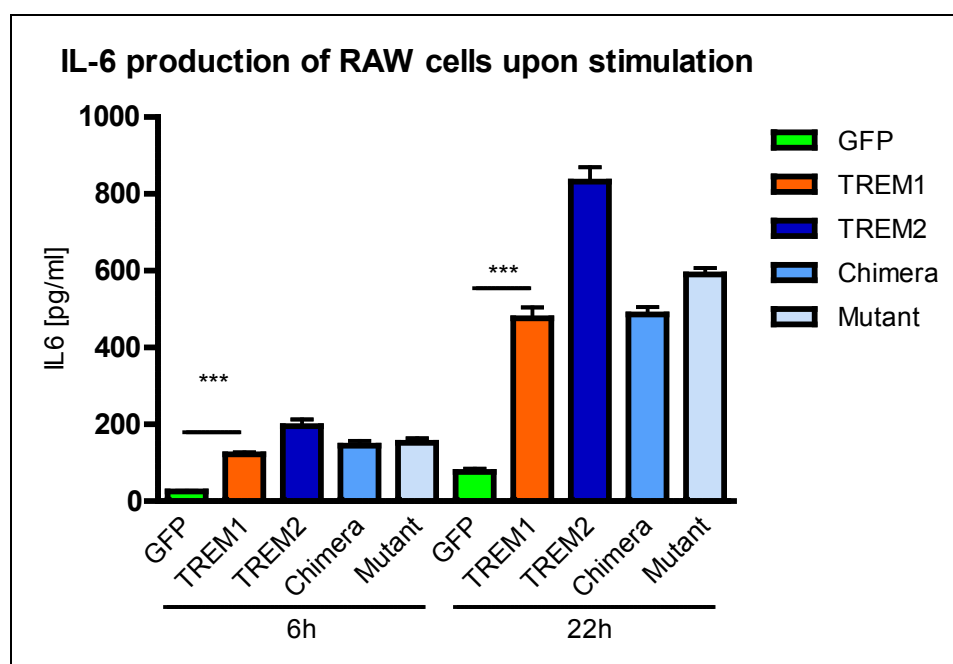
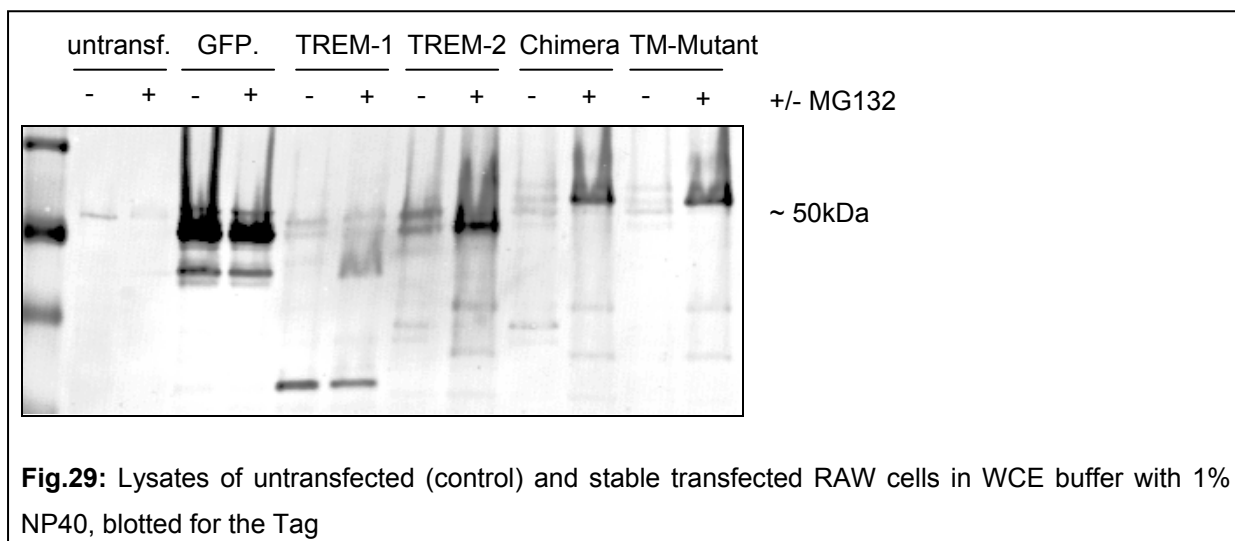


Fig.28: IL-6 production of stable transfected RAW cells in response to *S. pneumoniae* after 6h and 22h stimulation (Statistic analysis with Graphpad Prism: One way annova, Tukey-test)

The RAW cell lines, stable transfected with TREM-1, TREM-2 or the chimeric constructs, produce significantly more IL-6 (Fig. 28) This is surprising, since TREM-2 has been shown to be a negative regulator [80] of the inflammatory response. Nonetheless, these cells exhibit higher levels of proteasome activity (Fig 29) indicating cellular stress which could have an impact on pro-inflammatory cytokine production.

6.4.3. Solubility of the constructs expressed by RAW cells

Since TREM-2 has a singlepass transmembrane domain, which could result in unsolubility of the protein, three buffers with different stringency were used to lyse the cells and the TAP tag was detected by western blotting with an anti-Myc antibody. To check for proteasomal degradation all experiments were carried out with and without adding the proteasome inhibitor MG132 (Fig. 29).

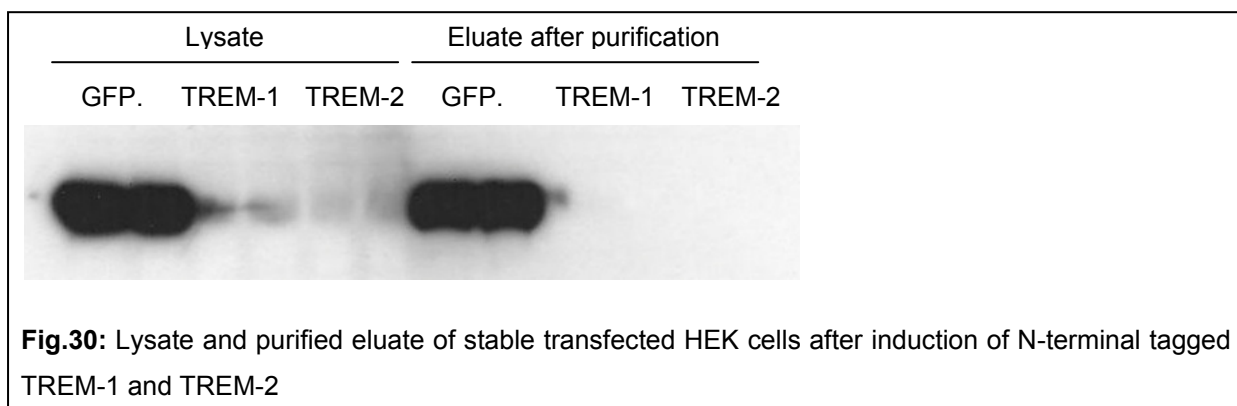


The same experiment was carried out with two more stringent buffers, one which contained 1% Chaps and a RIPA buffer. Since the result was exactly the same as with the NP40 buffer, the conclusion was that the NP40 buffer is sufficient to extract TREM-2 and the generated constructs.

Significantly, the TREM-2 constructs are obviously being degraded by the proteasome, since they are only detectable after addition of MG132. In case of the TREM-1 construct, the band which is visible at ~25kDa is most likely the Tag, which was cleaved off from the protein.

6.4.4. Pulldown experiment with Flp-InTM T-RExTM HEK 293 cells

One pulldown experiment was performed with the transfected HEK cells after induction of TREM-1 and TREM-2 expression with Doxycyclin, showing the same result which was already obtained from the experiments with RAW cells. There was only weak expression of the constructs, hardly detectable in the lysate and not enough for purification (Fig. 30).



7. Discussion

The surface receptor TREM-2 has been shown to be important for bone formation [98, 100] and brain homeostasis [89], as well as the regulation of immune responses [64]. Since downstream signaling of TREM-2 is still largely unknown, one important part of this work was the production of a TREM-2/DAP12 chimeric protein, consisting of the extracellular domain of TREM-2 fused to the intracellular domain of DAP12. Together with another TREM-2/DAP12 chimera carrying a mutation in the transmembrane domain, these constructs should be used as important controls in a TREM-2 pulldown in Flp-InTM T-RExTM HEK 293 cells and in RAW 264.7 cells.

The production of the fusion construct worked well in case of the mouse construct, and after several trials also for the human construct (Fig. 13). The introduction of a mutation in the transmembrane domain of DAP12 by site directed mutagenesis also worked immediately in case of the mouse chimera, but never for the human chimera. A sequence analysis of the constructs in gateway destination vectors, verified the fidelity of all constructs, except for the human fusion construct, which carried a point mutation in the TREM-2/DAP12 overlapping region. This point mutation was most likely introduced during the initial PCR steps and might be the reason why the site directed mutagenesis never worked.

Successfully transfected RAW 264.7 cells were selected by sorting them according to GFP expression (Fig. 26), HEK cells were selected for antibiotic resistance and survival. Unfortunately although the RAW cells expressed GFP, indicating that they were successfully transfected, they did not show any over-expression of TREM-2 in a FACS analysis (Fig. 27). A pulldown experiment with the HEK cells also showed only weak expression of TREM-2 and TREM-1 (Fig. 30). Since solubility turned out not to be the problem, we hypothesized that the constructs might get degraded. Indeed, cells pretreated with the proteasome inhibitor MG132 before lysis, expressed the TREM-2 constructs, as detected by western blotting with an antibody against the tag (Fig. 29). In case of TREM-1, MG132 did not change anything and it looks like the whole tag was simply cut off (Band at ~25kDa, Fig. 29).

Most membrane proteins are targeted to the membrane via an N-terminal localization signal, which is recognized by the signal recognition particle (SRP) immediately after synthesis, when the polypeptide is accessible [111]. Signal recognition by SRP is crucial for the targeting of signal-peptide bearing proteins to the endoplasmatic reticulum and for appropriate insertion, by GTP dependent interaction of the ribosome nascent chain-SRP complex with the SRP receptor and subsequent translocation through a channel called the translocon. The signal peptide is usually cleaved off after insertion [111, 112].

The decision to tag the pulldown constructs on the N-terminus was based on the fact that the intracellular domain of TREM-2 is tiny (~30 amino acids) and that the tag, especially the bigger TAP-tag, might interfere with possible interactions. Unluckily the tag most likely masked the localization signal in case of the TREM-2 constructs, leading to dislocation, and maybe accumulation in the cytoplasm, followed by proteasomal degradation. Since I will continue working on TREM-2 during my PhD, a C-terminal tagging of TREM-2 might help to overcome the problem of proteasomal degradation and will hopefully lead to success.

Another important part of this work was the characterization of TREM-2 deficient alveolar macrophages and their responses upon stimulation with *S. pneumoniae*.

In May 2010 Peng et al. showed that the phosphatase SHIP1 negatively regulates TREM-2 signaling [113]. TREM-2 ligation induces the recruitment of an activation complex including DAP12, DAP10, p85, Syk, and Grb, leading to activation of ERK1/2, Vav and AKT. Confocal analysis showed TREM-2 ligation to trigger the intracellular co-localization of SHIP1, F-actin, DAP10 and DAP12 in osteoclasts [113]. P85 is a regulatory subunit of class IA PI3-kinases, containing an SH2 domain which binds to phosphorylated tyrosines [114]. Engagement of P85 domains releases PI3-kinases from inhibition and triggers the contact with their lipid substrates in the membrane [114]. Upon membrane remodeling SHIP1 gets phosphorylated and recruited via its SH2 domain to the phosphorylated Tyr65 of DAP12 in the activation complex and converts it into an inhibitory one [113], by preventing additional recruitment of p85 or Syk to the complex, and thereby inhibits activating downstream signaling [113].

TREM-2 plays an important role in the pathogenesis of bacterial pneumonia. TREM-2 deficient mice show a significantly better survival in a survival study of streptococcal pneumonia (unpublished data, O. Sharif). Although TREM-2 has been published to be a phagocytic receptor for bacteria [95] and to be involved in phagocytosis of apoptotic neurons [96], a FACS based phagocytosis assay showed TREM-2 deficient AMs to have a higher phagocytic activity when challenged with *S. pneumoniae* (unpublished data, O. Sharif). Because this result was surprising it was verified in this work by using confocal microscopy (Fig. 11). The uptake of bacteria was perfectly visible and a staining of lysosomes showed partial co-localization of lysosomes and bacteria (data not shown).

Since the PI3- kinase/AKT pathway has been shown to be crucial for lipid remodeling [114] and therefore important for phagocytosis [57], we tested whether TREM-2^{-/-} AMs show differences in the activation of this pathway. Indeed, western blot analysis of WT and TREM-2 deficient AMs showed higher levels of AKT phosphorylation in the knockout cells, when challenged with *S. pneumoniae* (Fig. 13). When PI3-kinase gets activated, it phosphorylates phosphatidylinositol-4,5-bisphosphat (PIP₂) generating phosphatidylinositol-3,4,5-trisphosphat (PIP₃), a process which is antagonized by the PIP₃ phosphatase PTEN [115]. AKT and PDK1 are recruited to the membrane and bind to PIP₃ via their pleckstrin homology (PH) domain. AKT gets phosphorylated and activated by PDK1 [116] and mediates a variety of downstream signaling processes, inducing cell survival, growth, proliferation and angiogenesis [116]. PTEN is equally activated in TREM-2 deficient cells compared to WT (Fig. 14).

One important downstream target of pAKT is GSK-3 β which gets inactivated by phosphorylation of the N-terminal Ser9 [115]. GSK-3 β is a multifaceted kinase, mediating various processes including the regulation of TLR-mediated cytokine production [109]. LPS has been shown to activate AKT and inactivate GSK-3 β in human AMs, leading to nuclear accumulation and increased transcriptional activity of β -Catenin [117]. Higher levels of GSK-3 β phosphorylation could not be shown in this work (Fig. 15). This could be due to the different qualities of cell extracts, or the fact that GSK-3 β is regulated via kinases other than AKT, including protein kinase A (PKA) [118]. Together with a phagocytosis assay, showing that wortmannin, a PI3-kinase inhibitor, equalizes the phagocytic activity of WT and TREM-2^{-/-} AMs

(unpublished data, O. Sharif), and data from TREM-2 overexpressing HEK 293 cells showing lower levels of AKT phosphorylation, compared to normal HEK 293 cells when stimulated with *S. pneumoniae*, these data suggest that TREM-2 negatively regulates PI3-kinase activity. This result fits with the observation that the TREM-2/DAP12 complex recruits p85 in a SHIP1 dependent manner [113] and might also serve as an explanation for the increased cytokine responses of TREM-2 deficient macrophages in response to TLR stimuli [71].

Higher levels of phosphorylated ERK1/2 were also observed in TREM-2 deficient cells (Fig. 16), while p38 remained unaffected (Fig. 17). The same pattern has been shown for DAP12^{-/-} deficient macrophages, possibly suggesting that TREM-2 is the receptor responsible for this effect [110].

A killing assay performed with WT and TREM-2^{-/-} AMs showed no differences in the killing ability (Fig. 19), but taking into account that both cell types have the same time for the uptake of bacteria in this experiment and that TREM-2 deficient cells phagocytose more bacteria, this test might not be optimal to compare the killing ability of two cell types which differ in their phagocytic activity.

Since it has been published that TREM-2 is not only expressed on the cell surface, but shuttles between the trans-golgi network (TGN) and the cytoplasmic membrane in microglia [86], a staining of TREM-2 on AMs followed by confocal analysis was performed. Intracellular staining was performed by using Triton-X and TREM-2 was visible as accumulated dots (Fig. 20, left), similar to the puncta, which were seen by Prada et al. in microglia [86]. In their experiments 0.4% saponin was used, because they observed release of TREM-2 from the surface, when cells were treated with triton-X. This observation was not reproducible in this study. A surface staining showed TREM-2 to be homogeneously distributed on the cell surface (Fig. 20, right). Despite blocking with 10% serum the secondary antibody alone produced a quite strong unspecific signal (Fig. 21), but there was a clear difference visible between the control and cells which were treated with the anti-TREM-2 antibody (Fig. 20 and 21).

TREM-2^{-/-} osteoclasts [99] and DCs [82] show aberrant cell morphology, therefore the question was if TREM-2 deficiency would also affect the actin cytoskeleton of

AMs. No differences could be observed in cell size, shape or the distribution of F-actin between WT and TREM-2^{-/-} AMs, except for a generally lower F-actin signal in TREM-2^{-/-} cells, observed in two independent experiments. The confocal analysis performed in this work alone does not support the conclusion that there are lower F-actin levels in TREM-2^{-/-} AMs, but this could be tested for by FACS analysis in the future. Considering that SHIP1 negatively regulates TREM-2 signaling, that TREM-2 ligation leads to F-actin accumulation and that SHIP1^{-/-} BMDMs contain more F-actin, it would be worthy to follow up on this observation. .

8. Abbreviations

AEC	Alveolar epithelial cell
AM(s)	Alveolar macrophage(s)
<i>att</i> site	attachment site
BAL(F)	Broncho alveolar lavage (fluid)
BMDM	Bone marrow derived macrophage
CR	Complement receptor
CRP	C-reactive protein
DAG	Diacylglycerol
DAP10	DNAX activating protein of molecular mass 10 kDA
DAP12	DNAX activating protein of molecular mass 12 kDA, also called KARAP (killer cell activating receptor associated protein) or TYROBP (tyrosine kinase-binding protein)
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
ER	Endoplasmatic reticulum
ERK1/2	Extracellular signal regulated kinase1/2 or mitogen associated protein kinase (MAPK) p42/44
GFP	Green fluorescent protein
IFN γ	Interferon- γ
IgSF domain	Immunoglobuline superfamily-like domain
IHF	Integration host factor
I κ b	Inhibitors of NF κ b
IL-1	Interleukin-1
Int	λ Integrase
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MBL	Mannose binding lectin
MOI	Multiplicity of infection
NF κ b	Nuclear factor- κ b, Rel proteins
NLR	NOD-like receptor
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PH domain	Plextrin homology domain
PI3-Kinase	Phosphatidylinositol 3 Kinase
PIP2	Phosphatidylinositol-4,5-bisphosphat
PIP3	Phosphatidylinositol-3,4,5-trisphosphat
PKA	Protein kinase A
PKC	Protein kinase C
PLOSL	Polycystic lipomembranous osteodysplasia with sklerosing leukoencephalopathy or Nasu Hakola disease
Ply	Pneumolysin
PRR	Pattern recognition receptor
PTP	Phospho tyrosine phosphatase
SHIP	SH2-domain containing phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase
SH-tag	Streptavidin hemagglutinin-tag
SH2-domain	Src homology domain 2
<i>S.pneu.</i>	<i>Streptococcus pneumoniae</i>
Src	Src-kinase
TAP-tag	Tandem affinity purification-tag
TGN	Trans-golgi network
TIR-domain	Toll/Interleukin-1 receptor domain
TLR	Toll-like receptor
TLT	TREM-like transkript
TM-domain	Transmembrane domain
TM-mutant chimera	TREM-2/DAP12 chimera carrying a mutation in the TM-domain and therefore cannot associate anymore with receptors
TNF- α	Tumor necrosis factor- α
TREM-2	Triggering receptor expressed on myeloid cells-2
TREM-2-/-	TREM-2 knockout
TREM-1	Triggering receptor expressed on myeloid cells-2
WT	Wildtype
Xis	Excisionase

9. References

1. Turvey, S.E. and D.H. Broide, *Innate immunity*. J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S24-32.
2. Bonilla, F.A. and H.C. Oettgen, *Adaptive immunity*. J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S33-40.
3. Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition*. Annu Rev Immunol, 2002. **20**: p. 197-216.
4. Medzhitov, R. and C.A. Janeway, Jr., *Decoding the patterns of self and nonself by the innate immune system*. Science, 2002. **296**(5566): p. 298-300.
5. Lambrecht, B.N., *Alveolar macrophage in the driver's seat*. Immunity, 2006. **24**(4): p. 366-8.
6. Si-Tahar, M., L. Touqui, and M. Chignard, *Innate immunity and inflammation--two facets of the same anti-infectious reaction*. Clin Exp Immunol, 2009. **156**(2): p. 194-8.
7. Calbo, E. and J. Garau, *Of mice and men: innate immunity in pneumococcal pneumonia*. Int J Antimicrob Agents, 2010. **35**(2): p. 107-13.
8. Mizgerd, J.P., *Acute lower respiratory tract infection*. N Engl J Med, 2008. **358**(7): p. 716-27.
9. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity*. Cell, 2006. **124**(4): p. 783-801.
10. Pasare, C. and R. Medzhitov, *Toll-like receptors and acquired immunity*. Semin Immunol, 2004. **16**(1): p. 23-6.
11. Kumar, H., T. Kawai, and S. Akira, *Toll-like receptors and innate immunity*. Biochem Biophys Res Commun, 2009. **388**(4): p. 621-5.
12. Li, Q. and I.M. Verma, *NF-kappaB regulation in the immune system*. Nat Rev Immunol, 2002. **2**(10): p. 725-34.
13. Akira, S., *Pathogen recognition by innate immunity and its signaling*. Proc Jpn Acad Ser B Phys Biol Sci, 2009. **85**(4): p. 143-56.
14. Brown, G.D. and S. Gordon, *Immune recognition. A new receptor for beta-glucans*. Nature, 2001. **413**(6851): p. 36-7.
15. Taylor, P.R., et al., *Dectin-1 is required for beta-glucan recognition and control of fungal infection*. Nat Immunol, 2007. **8**(1): p. 31-8.
16. Meylan, E., J. Tschopp, and M. Karin, *Intracellular pattern recognition receptors in the host response*. Nature, 2006. **442**(7098): p. 39-44.
17. Medzhitov, R., *Recognition of microorganisms and activation of the immune response*. Nature, 2007. **449**(7164): p. 819-26.
18. Fritz, J.H., et al., *Nod-like proteins in immunity, inflammation and disease*. Nat Immunol, 2006. **7**(12): p. 1250-7.
19. Martinon, F. and J. Tschopp, *Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases*. Cell, 2004. **117**(5): p. 561-74.
20. Bryant, C. and K.A. Fitzgerald, *Molecular mechanisms involved in inflammasome activation*. Trends Cell Biol, 2009. **19**(9): p. 455-64.
21. Anevlavis, S. and D. Bouros, *Community acquired bacterial pneumonia*. Expert Opin Pharmacother, 2010. **11**(3): p. 361-74.
22. Lutfiyya, M.N., et al., *Diagnosis and treatment of community-acquired pneumonia*. Am Fam Physician, 2006. **73**(3): p. 442-50.
23. Mitchell, T.J., *Virulence factors and the pathogenesis of disease caused by Streptococcus pneumoniae*. Res Microbiol, 2000. **151**(6): p. 413-9.
24. Jonsson, S., et al., *Phagocytosis and killing of common bacterial pathogens of the lung by human alveolar macrophages*. J Infect Dis, 1985. **152**(1): p. 4-13.
25. Mitchell, A.M. and T.J. Mitchell, *Streptococcus pneumoniae: virulence factors and variation*. Clin Microbiol Infect, 2010. **16**(5): p. 411-8.
26. von Kockritz-Blickwede, M. and V. Nizet, *Innate immunity turned inside-out: antimicrobial defense by phagocyte extracellular traps*. J Mol Med, 2009. **87**(8): p. 775-83.
27. Boulnois, G.J., et al., *Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of Streptococcus pneumoniae*. Mol Microbiol, 1991. **5**(11): p. 2611-6.

28. Henriques-Normark, B. and S. Normark, *Commensal pathogens, with a focus on Streptococcus pneumoniae, and interactions with the human host*. Exp Cell Res, 2010. **316**(8): p. 1408-14.
29. Knapp, S., et al., *Lipoteichoic acid-induced lung inflammation depends on TLR2 and the concerted action of TLR4 and the platelet-activating factor receptor*. J Immunol, 2008. **180**(5): p. 3478-84.
30. Knapp, S., et al., *Toll-like receptor 2 plays a role in the early inflammatory response to murine pneumococcal pneumonia but does not contribute to antibacterial defense*. J Immunol, 2004. **172**(5): p. 3132-8.
31. Yoshimura, A., et al., *Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2*. J Immunol, 1999. **163**(1): p. 1-5.
32. Malley, R., et al., *Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection*. Proc Natl Acad Sci U S A, 2003. **100**(4): p. 1966-71.
33. Paterson, G.K. and T.J. Mitchell, *Innate immunity and the pneumococcus*. Microbiology, 2006. **152**(Pt 2): p. 285-93.
34. Schmeck, B., et al., *Pneumococci induced TLR- and Rac1-dependent NF-kappaB-recruitment to the IL-8 promoter in lung epithelial cells*. Am J Physiol Lung Cell Mol Physiol, 2006. **290**(4): p. L730-L737.
35. Mizgerd, J.P., et al., *Nuclear factor-kappaB p50 limits inflammation and prevents lung injury during Escherichia coli pneumonia*. Am J Respir Crit Care Med, 2003. **168**(7): p. 810-7.
36. Quinton, L.J., et al., *Functions and regulation of NF-kappaB RelA during pneumococcal pneumonia*. J Immunol, 2007. **178**(3): p. 1896-903.
37. Knapp, S., et al., *Alveolar macrophages have a protective antiinflammatory role during murine pneumococcal pneumonia*. Am J Respir Crit Care Med, 2003. **167**(2): p. 171-9.
38. Biffl, W.L., et al., *Interleukin-6 delays neutrophil apoptosis via a mechanism involving platelet-activating factor*. J Trauma, 1996. **40**(4): p. 575-8; discussion 578-9.
39. Kaplan, M.H. and J.E. Volanakis, *Interaction of C-reactive protein complexes with the complement system. I. Consumption of human complement associated with the reaction of C-reactive protein with pneumococcal C-polysaccharide and with the choline phosphatides, lecithin and sphingomyelin*. J Immunol, 1974. **112**(6): p. 2135-47.
40. Li, M.O. and R.A. Flavell, *Contextual regulation of inflammation: a duet by transforming growth factor-beta and interleukin-10*. Immunity, 2008. **28**(4): p. 468-76.
41. Mosser, D.M. and X. Zhang, *Interleukin-10: new perspectives on an old cytokine*. Immunol Rev, 2008. **226**: p. 205-18.
42. Zhang, X., et al., *Coactivation of Syk kinase and MyD88 adaptor protein pathways by bacteria promotes regulatory properties of neutrophils*. Immunity, 2009. **31**(5): p. 761-71.
43. Geissmann, F., et al., *Development of monocytes, macrophages, and dendritic cells*. Science, 2010. **327**(5966): p. 656-61.
44. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation*. Nat Rev Immunol, 2008. **8**(12): p. 958-69.
45. Gordon, S., *Alternative activation of macrophages*. Nat Rev Immunol, 2003. **3**(1): p. 23-35.
46. Mantovani, A., et al., *The chemokine system in diverse forms of macrophage activation and polarization*. Trends Immunol, 2004. **25**(12): p. 677-86.
47. Varin, A. and S. Gordon, *Alternative activation of macrophages: immune function and cellular biology*. Immunobiology, 2009. **214**(7): p. 630-41.
48. Martinez, F.O., L. Helming, and S. Gordon, *Alternative activation of macrophages: an immunologic functional perspective*. Annu Rev Immunol, 2009. **27**: p. 451-83.
49. Thepen, T., N. Van Rooijen, and G. Kraal, *Alveolar macrophage elimination in vivo is associated with an increase in pulmonary immune response in mice*. J Exp Med, 1989. **170**(2): p. 499-509.
50. Takabayshi, K., et al., *Induction of a homeostatic circuit in lung tissue by microbial compounds*. Immunity, 2006. **24**(4): p. 475-87.
51. Aderem, A., *How to eat something bigger than your head*. Cell, 2002. **110**(1): p. 5-8.
52. Allen, L.A. and A. Aderem, *Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages*. J Exp Med, 1996. **184**(2): p. 627-37.
53. Touret, N., P. Paroutis, and S. Grinstein, *The nature of the phagosomal membrane: endoplasmic reticulum versus plasmalemma*. J Leukoc Biol, 2005. **77**(6): p. 878-85.

54. Greenberg, S. and S. Grinstein, *Phagocytosis and innate immunity*. Curr Opin Immunol, 2002. **14**(1): p. 136-45.
55. Fitzer-Attas, C.J., et al., *Fcgamma receptor-mediated phagocytosis in macrophages lacking the Src family tyrosine kinases Hck, Fgr, and Lyn*. J Exp Med, 2000. **191**(4): p. 669-82.
56. Crowley, M.T., et al., *A critical role for Syk in signal transduction and phagocytosis mediated by Fcgamma receptors on macrophages*. J Exp Med, 1997. **186**(7): p. 1027-39.
57. Marshall, J.G., et al., *Restricted accumulation of phosphatidylinositol 3-kinase products in a plasmalemmal subdomain during Fc gamma receptor-mediated phagocytosis*. J Cell Biol, 2001. **153**(7): p. 1369-80.
58. Massol, P., et al., *Fc receptor-mediated phagocytosis requires CDC42 and Rac1*. EMBO J, 1998. **17**(21): p. 6219-29.
59. Caron, E. and A. Hall, *Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases*. Science, 1998. **282**(5394): p. 1717-21.
60. Allcock, R.J., et al., *The human TREM gene cluster at 6p21.1 encodes both activating and inhibitory single IgV domain receptors and includes NKp44*. Eur J Immunol, 2003. **33**(2): p. 567-77.
61. Ford, J.W. and D.W. McVicar, *TREM and TREM-like receptors in inflammation and disease*. Curr Opin Immunol, 2009. **21**(1): p. 38-46.
62. Klesney-Tait, J., I.R. Turnbull, and M. Colonna, *The TREM receptor family and signal integration*. Nat Immunol, 2006. **7**(12): p. 1266-73.
63. Barrow, A.D., et al., *Cutting edge: TREM-like transcript-1, a platelet immunoreceptor tyrosine-based inhibition motif encoding costimulatory immunoreceptor that enhances, rather than inhibits, calcium signaling via SHP-2*. J Immunol, 2004. **172**(10): p. 5838-42.
64. Sharif, O. and S. Knapp, *From expression to signaling: roles of TREM-1 and TREM-2 in innate immunity and bacterial infection*. Immunobiology, 2008. **213**(9-10): p. 701-13.
65. Kelker, M.S., et al., *Crystal structure of human triggering receptor expressed on myeloid cells 1 (TREM-1) at 1.47 Å*. J Mol Biol, 2004. **342**(4): p. 1237-48.
66. Kelker, M.S., E.W. Debler, and I.A. Wilson, *Crystal structure of mouse triggering receptor expressed on myeloid cells 1 (TREM-1) at 1.76 Å*. J Mol Biol, 2004. **344**(5): p. 1175-81.
67. Radaev, S., et al., *Crystal structure of the human myeloid cell activating receptor TREM-1*. Structure, 2003. **11**(12): p. 1527-35.
68. Turnbull, I.R. and M. Colonna, *Activating and inhibitory functions of DAP12*. Nat Rev Immunol, 2007. **7**(2): p. 155-61.
69. Lanier, L.L., *DAP10- and DAP12-associated receptors in innate immunity*. Immunol Rev, 2009. **227**(1): p. 150-60.
70. Feng, J., M.E. Call, and K.W. Wucherpfennig, *The assembly of diverse immune receptors is focused on a polar membrane-embedded interaction site*. PLoS Biol, 2006. **4**(5): p. e142.
71. Hamerman, J.A., et al., *Cutting edge: inhibition of TLR and FcR responses in macrophages by triggering receptor expressed on myeloid cells (TREM)-2 and DAP12*. J Immunol, 2006. **177**(4): p. 2051-5.
72. Hamerman, J.A. and L.L. Lanier, *Inhibition of immune responses by ITAM-bearing receptors*. Sci STKE, 2006. **2006**(320): p. re1.
73. Barrow, A.D. and J. Trowsdale, *You say ITAM and I say ITIM, let's call the whole thing off: the ambiguity of immunoreceptor signalling*. Eur J Immunol, 2006. **36**(7): p. 1646-53.
74. Chu, C.L., et al., *Increased TLR responses in dendritic cells lacking the ITAM-containing adapters DAP12 and FcRgamma*. Eur J Immunol, 2008. **38**(1): p. 166-73.
75. Nasu, T., Y. Tsukahara, and K. Terayama, *A lipid metabolic disease-"membranous lipodystrophy"-an autopsy case demonstrating numerous peculiar membrane-structures composed of compound lipid in bone and bone marrow and various adipose tissues*. Acta Pathol Jpn, 1973. **23**(3): p. 539-58.
76. Hakola, H.P., O.H. Jarvi, and P. Sourander, *Osteodysplasia polycystica hereditaria combined with sclerosing leucoencephalopathy, a new entity of the dementia praesentis group*. Acta Neurol Scand, 1970. **46**: p. Suppl 43:79+.
77. Bianchin, M.M., et al., *Nasu-Hakola disease (polycystic lipomembranous osteodysplasia with sclerosing leucoencephalopathy--PLOS): a dementia associated with bone cystic lesions. From clinical to genetic and molecular aspects*. Cell Mol Neurobiol, 2004. **24**(1): p. 1-24.
78. Kaneko, M., et al., *Nasu-Hakola disease: The first case reported by Nasu and review*. Neuropathology, 2010.

79. Paloneva, J., et al., *CNS manifestations of Nasu-Hakola disease: a frontal dementia with bone cysts*. Neurology, 2001. **56**(11): p. 1552-8.
80. Turnbull, I.R., et al., *Cutting edge: TREM-2 attenuates macrophage activation*. J Immunol, 2006. **177**(6): p. 3520-4.
81. Woodruff, P.G., et al., *Alveolar macrophage recruitment and activation by chronic second hand smoke exposure in mice*. COPD, 2009. **6**(2): p. 86-94.
82. Kiialainen, A., et al., *Transcript profiles of dendritic cells of PLOSL patients link demyelinating CNS disorders with abnormalities in pathways of actin bundling and immune response*. J Mol Med, 2007. **85**(9): p. 971-83.
83. Kiialainen, A., et al., *Dap12 and Trem2, molecules involved in innate immunity and neurodegeneration, are co-expressed in the CNS*. Neurobiol Dis, 2005. **18**(2): p. 314-22.
84. Thrash, J.C., B.E. Torbett, and M.J. Carson, *Developmental regulation of TREM2 and DAP12 expression in the murine CNS: implications for Nasu-Hakola disease*. Neurochem Res, 2009. **34**(1): p. 38-45.
85. Piccio, L., et al., *Identification of soluble TREM-2 in the cerebrospinal fluid and its association with multiple sclerosis and CNS inflammation*. Brain, 2008. **131**(Pt 11): p. 3081-91.
86. Prada, I., et al., *Triggering receptor expressed in myeloid cells 2 (TREM2) trafficking in microglial cells: continuous shuttling to and from the plasma membrane regulated by cell stimulation*. Neuroscience, 2006. **140**(4): p. 1139-48.
87. Sessa, G., et al., *Distribution and signaling of TREM2/DAP12, the receptor system mutated in human polycystic lipomembraneous osteodysplasia with sclerosing leukoencephalopathy dementia*. Eur J Neurosci, 2004. **20**(10): p. 2617-28.
88. Chen, L.C., et al., *Regulation of TREM expression in hepatic macrophages and endothelial cells during acute endotoxemia*. Exp Mol Pathol, 2008. **84**(2): p. 145-55.
89. Neumann, H. and K. Takahashi, *Essential role of the microglial triggering receptor expressed on myeloid cells-2 (TREM2) for central nervous tissue immune homeostasis*. J Neuroimmunol, 2007. **184**(1-2): p. 92-9.
90. Lagler, H., et al., *TREM-1 activation alters the dynamics of pulmonary IRAK-M expression in vivo and improves host defense during pneumococcal pneumonia*. J Immunol, 2009. **183**(3): p. 2027-36.
91. Takegahara, N., et al., *Plexin-A1 and its interaction with DAP12 in immune responses and bone homeostasis*. Nat Cell Biol, 2006. **8**(6): p. 615-22.
92. Radhakrishnan, S., et al., *TREM-2 mediated signaling induces antigen uptake and retention in mature myeloid dendritic cells*. J Immunol, 2008. **181**(11): p. 7863-72.
93. Whittaker, G.C., et al., *The linker for activation of B cells (LAB)/non-T cell activation linker (NTAL) regulates triggering receptor expressed on myeloid cells (TREM)-2 signaling and macrophage inflammatory responses independently of the linker for activation of T cells*. J Biol Chem, 2010. **285**(5): p. 2976-85.
94. Helming, L., et al., *Essential role of DAP12 signaling in macrophage programming into a fusion-competent state*. Sci Signal, 2008. **1**(43): p. ra11.
95. N'Diaye, E.N., et al., *TREM-2 (triggering receptor expressed on myeloid cells 2) is a phagocytic receptor for bacteria*. J Cell Biol, 2009. **184**(2): p. 215-23.
96. Hsieh, C.L., et al., *A role for TREM2 ligands in the phagocytosis of apoptotic neuronal cells by microglia*. J Neurochem, 2009. **109**(4): p. 1144-56.
97. Piccio, L., et al., *Blockade of TREM-2 exacerbates experimental autoimmune encephalomyelitis*. Eur J Immunol, 2007. **37**(5): p. 1290-301.
98. Cella, M., et al., *Impaired differentiation of osteoclasts in TREM-2-deficient individuals*. J Exp Med, 2003. **198**(4): p. 645-51.
99. Paloneva, J., et al., *DAP12/TREM2 deficiency results in impaired osteoclast differentiation and osteoporotic features*. J Exp Med, 2003. **198**(4): p. 669-75.
100. Park-Min, K.H., et al., *IL-10 suppresses calcium-mediated costimulation of receptor activator NF-kappa B signaling during human osteoclast differentiation by inhibiting TREM-2 expression*. J Immunol, 2009. **183**(4): p. 2444-55.
101. Hashiguchi, M., et al., *Triggering receptor expressed on myeloid cell-like transcript 2 (TLT-2) is a counter-receptor for B7-H3 and enhances T cell responses*. Proc Natl Acad Sci U S A, 2008. **105**(30): p. 10495-500.
102. Chapoval, A.I., et al., *B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production*. Nat Immunol, 2001. **2**(3): p. 269-74.

103. Daws, M.R., et al., *Pattern recognition by TREM-2: binding of anionic ligands*. J Immunol, 2003. **171**(2): p. 594-9.
104. Quan, D.N., et al., *TREM-2 binds to lipooligosaccharides of Neisseria gonorrhoeae and is expressed on reproductive tract epithelial cells*. Mucosal Immunol, 2008. **1**(3): p. 229-38.
105. Stefano, L., et al., *The surface-exposed chaperone, Hsp60, is an agonist of the microglial TREM2 receptor*. J Neurochem, 2009. **110**(1): p. 284-94.
106. Wurch, T., F. Lestienne, and P.J. Pauwels, *A modified overlap extension PCR method to create chimeric genes in the absence of restriction enzymes*. Biotechnology Techniques, 1998. **12**(9): p. 653-657.
107. Lanier, L.L., et al., *Association of DAP12 with activating CD94/NKG2C NK cell receptors*. Immunity, 1998. **8**(6): p. 693-701.
108. Glatter, T., et al., *An integrated workflow for charting the human interaction proteome: insights into the PP2A system*. Mol Syst Biol, 2009. **5**: p. 237.
109. Martin, M., et al., *Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3*. Nat Immunol, 2005. **6**(8): p. 777-84.
110. Hamerman, J.A., et al., *Enhanced Toll-like receptor responses in the absence of signaling adaptor DAP12*. Nat Immunol, 2005. **6**(6): p. 579-86.
111. Janda, C.Y., et al., *Recognition of a signal peptide by the signal recognition particle*. Nature, 2010. **465**(7297): p. 507-10.
112. Nagai, K., et al., *Structure, function and evolution of the signal recognition particle*. EMBO J, 2003. **22**(14): p. 3479-85.
113. Peng, Q., et al., *TREM2- and DAP12-dependent activation of PI3K requires DAP10 and is inhibited by SHIP1*. Sci Signal, 2010. **3**(122): p. ra38.
114. Vanhaesebroeck, B., et al., *The emerging mechanisms of isoform-specific PI3K signalling*. Nat Rev Mol Cell Biol, 2010. **11**(5): p. 329-41.
115. Tamguney, T. and D. Stokoe, *New insights into PTEN*. J Cell Sci, 2007. **120**(Pt 23): p. 4071-9.
116. Manning, B.D. and L.C. Cantley, *AKT/PKB signaling: navigating downstream*. Cell, 2007. **129**(7): p. 1261-74.
117. Monick, M.M., et al., *Lipopolysaccharide activates Akt in human alveolar macrophages resulting in nuclear accumulation and transcriptional activity of beta-catenin*. J Immunol, 2001. **166**(7): p. 4713-20.
118. Jope, R.S. and G.V. Johnson, *The glamour and gloom of glycogen synthase kinase-3*. Trends Biochem Sci, 2004. **29**(2): p. 95-102.

Curriculum Vitae

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Practice

August 2000	Internship, Boehringer Ingelheim Austria GmbH (department for molecularbiology)
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Appendix

Supplementary protocols

SOP: Inoculum *S. pneumonia*

S. Knapp, 14.02. 2007, Version01

A *Start: Strept pneumoniae ATCC 6303*

- 1 Media TH (Todd Hewitt) 50 ml and 100 ml (Waltraud Schmid, order one week in advance)

label: name, species of bacteria, date.

- 2 Bacteria (-70°C 3P: Refco Freezer, workspace on the right hand side, second shelf from the bottom): S.pneu ATCC 6303, spleen stock

In ML-II room (3P):

- 3 clean the bench: first with water, and then with 70 % alcohol. Let alcohol dry.

Clean Eye gloom in flame, let cool down. Open the flask with TH-media lightly (don't remove the lid)

- 4 Make sure that the bacteria are slightly thawed (shake it carefully). Open the tube with the bacteria, and load a "glassball" on the Eye. Remove the lid of the TH-media (50 ml tube). Put the "glassball" into the media without touching the border of the tube. Close the lid. Close the TH-Media.

- 5 Take the TH-media with the glassball in the 37 °C room. Then open the lid slightly. Incubate for 16 hours (over night). Store the second flask with the 100 ml TH-media also in the 37 °C room (label the flask as described). Put the stock with bacteria back in the -70°C.

- 6 Clean Eye gloom in flame again and clean the bench with 70 % alcohol.

B *next day: Expansion of the Bakteria: 6h*

- 1 Take the bacteria suspension (50 ml TH-media which is now unclear) and the fresh 100 ml TH-media from the 37 °C room and put it into the ML-II lab.

Clean the bench like described before.

Take 1 ml of the bacterial suspension (sterile with Gilson-pipette) and transfer it into the 100 ml TH-media. Close the lid and put it to the 37 °C room. Open the lid slightly there.

- 2 Grow the culture for 6 hours at 37 °C.
- 3 Clean the bench like described. Dispose 50 ml of TH-media.

C *measuring of the OD, dilute the inoculum, exposing*

- 1 Turn on the *Spectrophotometer 3P*, *prewarm* (20-30 minutes), adjust to 620nm and single measurement.
- 2 Clean the bench. Take the bacteria to the ML-II room.

Shake the bacteria suspension slightly (this is for a homogeneous distribution of the bacteria).

Take 1 ml (sterile) and add in to a cuvette. Take the second cuvette and fill it with water as blank.

- 3 Place the cuvettes in the photometer. Determine zero by measuring the blank first.

Measure then the bacteria, OD should be between 0,8 and 0,9 ideally.

- 4 In the case that OD is only 0,7 it is necessary to put the TH-media again to 37°C for approximately 20 minutes. Repeat this process twice or three times.

When OD has reached the optimal amount, continue with step 5.

- 5 take the 100 ml of bacterial suspension and put it to two 50 ml Greiner Tubes (each tube 50 ml), centrifuge at 4000rpm, 15 min, 4°C

- 6 Discard supernatant slightly (pellet is soft and liquid)

Add in sterile NaCl to have a final volume of 50 ml. Centrifuge like described in step 5.

- 7 Discard supernatant and collect the pellets in one tube.

Add in again 50 ml NaCl and centrifuge again.

- 8 Discard supernatant and add in 10 ml sterile NaCl (bacteria stock)

- 9 produce dilutions: in two Steps: 1:1000 dilution:

A. 1: 20 dilution: 1ml bacteria stock

19ml NaCl

B 1: 50 dilution: 1ml of A
 49ml NaCl

10 it depends on the value of OD (if OD is higher than 0,9) dilute additionally 1:5 (1ml von B + 4ml NaCl)

11 final dilution (either 9B or 10) put on ice.

This final bacteria suspension is needed for 1. the mice and

2. has to be exposed to Bloodagar plates, to determine the real concentration of bacteria.

12 Determination of the bacterial density: 5 small tubes (Micronic) with 180 µl of sterile NaCl each, label 1 to 5. Add in 20 µl of the bacterial suspension (that means 9 B or 10) in tube # 1, mix it properly. Abolish the pipette tip and take a new one. Add in 20µl of the solution in tube #1 to tube # 2, mix it properly, and continue with this procedure till tube #5.

13 take 7 Bloodagar plates and label them with date, name and dilutionfactor: make the following dilutions: undiluted, 2×10^{-3} , 2×10^{-4} und 2×10^{-5} . Take 50 µl of the undiluted solution (from 9 B or 10) and pipette it on the plate and dispense it over the plate with a sterile glass scoop. Take 50 µl of tube #3 and pipette it on both of the two plates and proceed in the same way.

Continue with this processing for the remaining plates.

Put the plates in the 37°C room (with the lid down).

Count CFUs the next day and write down the number.

D Disposal of the bacteria and solutions

1 The remaining bacteria supspension as well as the supernatant have to be put to a glasbottle and pooled. When the work is finished it is necessary to autoclave (20 minutes, 120°C) and dispose as liquid labwaste.

2 Put the bloodagar plates after the counting in a blue Meteka-bag.

Protocol for heat shock transformation of *E. coli*

- 1) competent cells were defrosted on ice and the plasmid DNA was added (50ng is sufficient, as a control PUC19 was used)
- 2) incubation on ice for 30min, heat shock at 42°C for 45sec, again ice for 2min
- 3) added 200 – 300µl SOC media (or LB) and incubated for 1h on a shaker
- 4) bacteria were plated out on LB-agar (supplemented with the appropriate antibiotics) and incubated over night at 37°C

Protocol for heat inactivated bacteria

Molecular biology methods, SOP#8, Version01

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Approved: Sylvia Knapp

Materials

Buffers:

LB-Medium: 1% Tryptone (Sigma T9410); 0,5% Yeast Extract (Sigma Y0375); 1% NaCl (Merck 1.06404) in A.dest.

Additionally required reagents, disposables and devices:

A.dest. (Mayrhofer Pharmazeutika; 15.533)

0,9% NaCl solution (Mayerhofer Pharmazeutika; 15.553)

Columbia blood agar plates (Bio Merieux; 43041)

Latex gloves DermaClean® (Ansell; PFC4303971/30001); required for the whole procedure!!!

Needle, sterilized by flame (Lab VI)

sterilized Glassware, several

Gilson Pipetman P20

Gilson Pipetman P100

Gilson Pipetman P1000

1-20µl Bevelled Filter Tip (StarLab; S1120-1810)

1-100µl Bevelled Filter Tip (StarLab; S1120-1840)

101-1000µl Filter Tips (StarLab; S1126-7810)

10ml Stripette® (Costar®; 4101)

Pipetboy accu, Integra Biosciences

15ml PP tubes Cellstar® (Greiner ; 188-261)

1,5ml Mixed Colors Flat Cap Microcentrifuge Tubes „epi“ (StarLab; S1615-5599)

Cuvettes; greiner bio-one (nr.613101)

Centrifuge Rotanta 460R; Hettich

Vortex

Balance METTLER PJ3000, use for 0,01-1000g

Spectrophotometer U-2000; Hitachi

Working Procedure

- inoculate bacteria of interest on a blood agar plate and strike to the colony (see classical microbiological literature)
- incubate over night at 37°C
- put 20ml LB in a 100ml Glass Erlenmeyer flask
- take 6 single colonies with a sterile needle and inoculate 20ml LB-Medium
- incubate 1,5-2,5h/37°C/200rpm
- take 1ml and measure OD at 620nm (take water for blank)
- if OD ~1 put 9ml in a 15ml Falcon tube and centrifuge 4°C/4000rpm/12min (if not grow until OD~1)
- discard supernatant and resuspend pellet in 10ml NaCl solution
- centrifuge again 4°C /4000rpm/12min
- repeat centrifugation step
- do colony count → see SOP#15
- heat inactivate remaining suspension in a waterbath at 65°C/30min
- store heat inactivated solution at -20°C

For non fastidious bacteria (as *Streptococcus pneumoniae* and *Haemophilus influenzae*) please refer to classical bacteriological literature for changes in working procedure (especially growing media and circumstances)

Protocol for FITC labeling heat killed bacteria

- 1) prepare fresh 0.1M NaHCO₃ at pH 9.0 (adjusted with NaOH)
- 2) thaw heat killed bacteria (stored in 50ml falcon at -20°C)
- 3) spin down: 2800rpm, 8min, spill supernatant
- 4) add 10ml 0.1M NaHCO₃ to the pellet
- 5) spin down again
- 6) meanwhile dissolve 4mg of FITC (Sigma, F7250) in 1ml 0.1M NaHCO₃ and filter solution through a fine mesh (FACS tubes with blue filter)
- 7) dilute FITC solution to a concentration of 0.2mg/ml with 0.1M NaHCO₃
- 8) resuspend bacteria in all the 20ml of FITC solution
- 9) incubate for 1h at 37°C in shaking water bath
- 10) spin down and trash supernatant
- 11) wash 1x with PBS and spin again
- 12) resuspend to get a final concentration of 10E9 CFU/ml and aliquot in to 2ml tubes (1ml per tube)